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(REV. 11-94)U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

8607-023-999

**TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)**INTERNATIONAL APPLICATION NO.
PCT/CA97/00969INTERNATIONAL FILING DATE
December 15, 1997PRIORITY DATE CLAIMED
December 13, 1996, January 24, 1997,
April 24, 1997TITLE OF INVENTION
CLONED GLUCAGON-LIKE PEPTIDE 2 RECEPTORS

512 Rec'd PCT/PTO 1 4 JUN 1999

APPLICANT(S) FOR DO/EO/US
Allelix Biopharmaceuticals Inc.

Applicant herewith submits to the United States Designated/ Elected Office (DO/EO/US) the following items under 35 U.S.C. 371:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
- ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
- ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as published (35 U.S.C. 371(c)(2))
- a. ☒ is transmitted herewith (required only if not transmitted by the international Bureau).
- b. ☐ has been transmitted by the International Bureau.
- c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
- a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
- b. ☐ have been transmitted by the International Bureaus.
- c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
- d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 37(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
- ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☐ A Preliminary Amendment.

17. ☒ The U.S. National Fee (35 U.S.C. 371(c)(1)) and other fees as follows:

CLAIMS				
(1)FOR	(2)NUMBER FILED	(3)NUMBER EXTRA	(4)RATE	(5)CALCULATIONS
TOTAL CLAIMS	19 - 20	0	X \$ 18.00	\$ 0.00
INDEPENDENT CLAIMS	6 - 3	3	X \$ 78.00	234.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$ 260.00	
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)): CHECK ONE BOX ONLY				
<input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) \$ 670				\$
<input type="checkbox"/> No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) \$ 760				
<input checked="" type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$ 970				\$ 970.00
<input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2) to (4) \$ 96				
<input type="checkbox"/> Filing with EPO or JPO search report \$ 840				
Surcharge of \$130.00 for furnishing the National fee or oath or declaration later than 20 30 mos. from the earliest claimed priority date (37 CFR 1.492(e)).				\$ 0.00
TOTAL OF ABOVE CALCULATIONS				= 1,204.00
Reduction by 1/2 for filing by small entity, if applicable. Affidavit must be filed also. (Note 37 CFR 1.9, 1.27, 1.28).				- \$ 0.00
SUBTOTAL				= 1,204.00
Processing fee of \$130.00 for furnishing the English Translation later than 20 30 mos. from the earliest claimed priority date (37 CFR 1.492(f)).				+
0				TOTAL FEES ENCLOSED \$ 1,204.00

- a. ☐ A check in the amount of \$__ to cover the above fees is enclosed.
- b. ☒ Please charge Deposit Account No. 16-1150 in the amount of \$1,204.00 to cover the above fees. A copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 16-1150. A copy of this sheet is enclosed.

18. ☐ Other instructions
n/a19. ☒ All correspondence for this application should be mailed to
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NEW YORK, NEW YORK 10036-271120. ☒ All telephone inquiries should be made to (212) 790-6431Thomas E. Friebe
NAME

SIGNATURE

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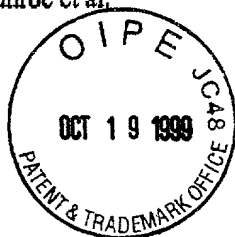
REGISTRATION NUMBER

June 14, 1999
DATE

#3

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: ☒ Application of: Munroe et al.
☐ Patent of:



☒ Serial No.: 09/331,127
☐ Patent No.:

Group Art Unit:

☒ Filed: June 14, 1999
☐ Issued:

Examiner: To Be Assigned

For: CLONED GLUCAGON-LIKE PEPTIDE-2
 RECEPTORS

Attorney Docket No.:
 8607-023-999

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
 [37 CFR 1.9(f) and 1.27(c)] - Small Business Concern

Assistant Commissioner for Patents
 Washington, D.C. 20231

Sir:

I hereby declare that I am

- ☐ the owner of the small business concern identified below:
☒ an official of the small business concern empowered to act in behalf of
 the concern identified below:

Name of concern Allelix Biopharmaceuticals Inc.

Address of concern 6850 Goreway Drive

Mississauga, Ontario, Canada L4V 1V7

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the person employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern and/or there is an obligation under contract or law by the inventor(s) to convey rights to the small business concern with regard to the invention, entitled **METHODS OF ENHANCING FUNCTIONING OF THE LARGE INTESTINE** by inventor(s) Donald G. Munroe, Ashwani K. Gupta, Tejal B. Vyas, Kirk McCallum, and Ermei Fan, described in

- ☐ the specification filed herewith
☒ application serial no.09/331,127 filed June 14, 1999
☐ patent no. issued

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who could not qualify

NY2-985346.1

as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e).

FULL NAME _____

ADDRESS _____

☐ INDIVIDUAL

☐ SMALL BUSINESS CONCERN

☐ NONPROFIT ORGANIZATION

FULL NAME _____

ADDRESS _____

☐ INDIVIDUAL

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I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. [37 CFR 1.28 (b)]

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, and patent issuing thereon, or any patent to which this verified statement is directed.

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Name of person signing Graham Strachan

Title of person other than owner President and Chief Executive Officer

Address of person signing Allelix Biopharmaceuticals Inc.

6850 Goreway Drive, Mississauga, Ontario, Canada L4V 1V7

Signature _____

Date _____

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

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12/PRTS

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WO 98/25955

PCT/CA97/00969

CLONED GLUCAGON-LIKE PEPTIDE-2 RECEPTORS

FIELD OF THE INVENTION

The invention is in the field of molecular biology. It relates, more particularly, to cloned glucagon-like peptide 2 receptors and their use in drug screening and related applications.

BACKGROUND TO THE INVENTION

Glucagon-like peptide-2 (GLP-2) is a 33 amino acid peptide, which is expressed in a tissue determined fashion from the pleiotrophic glucagon gene and is highly related in terms of amino acid sequence to glucagon and Glucagon-like peptide-1 (GLP-1). Mammalian forms of GLP-2 are highly conserved: for example, the human and degu (a south American rodent) forms differ by one and three amino acids respectively from rat GLP-2. Recently it was demonstrated that GLP-2 is an intestinotrophic peptide hormone; when given exogenously, GLP-2 can produce a marked increase in the proliferation of small intestinal epithelium of the test mice (Drucker et al, (1996) PNAS, 93:7911-7961). More recently, GLP-2 has been shown to increase D-Glucose maximal transport rate across the intestinal basolateral membrane (Cheeseman and Tseng: American Journal of Physiology (1996) 271:G477-G482).

To accelerate research into gastrointestinal biology and development of drugs useful in the treatment of various medical conditions including gastrointestinal disorders, it would be useful to provide the receptor through which the effects of GLP-2 are mediated.

SUMMARY OF THE INVENTION

The GLP-2 receptor has now been cloned and characterized. Accordingly, the present invention provides an isolated polynucleotide encoding a GLP-2 receptor, particularly including mammalian forms and homologs thereof such as, in specific embodiments, the rat and human forms. In aspects of the invention, polynucleotide coding for a GLP-2 receptor is utilized for expression to obtain functional receptor protein and, in optionally labelled form, for further gene cloning to identify structurally related receptor proteins. In related aspects of the invention, anti-sense versions of GLP-2 receptor-encoding polynucleotides and fragments thereof are obtained and utilized to regulate GLP-2 receptor expression.

In another of its aspects, the invention provides GLP-2 receptor as a product of recombinant production in a cellular host. In related aspects, there are provided recombinant host cells that express GLP-2 receptor, as well as receptor-bearing membranes derived from such cells, and expression constructs in which polynucleotide coding for the GLP-2 receptor is linked to expression controls functional in the selected host cell.

In another of its aspects, the GLP-2 receptor is utilized in a chemicals screening program to identify GLP-2 receptor ligands. This method comprises the steps of incubating the candidate ligand with a GLP-2 receptor-producing cell of the present invention, or with a membrane preparation derived therefrom, and then measuring whether, or the extent to which, binding has occurred. Using cells that express a GLP-2 receptor coupled functionally to a second messenger system, such binding can be determined indirectly, to reveal ligand against activity, by detecting an appropriate reporter.

In another of its aspects, the invention provides antibodies directed to the GLP-2 receptor, for use for example in diagnostic procedures.

The invention is further described with reference to the following drawings in which:

BRIEF REFERENCE TO THE DRAWINGS

Figure 1 discloses a cDNA sequence (SEQ ID NO: 1), nucleotides 137-1789 of which encode the rat GLP-2 receptor wherein ambiguous base pairs are indicated using the standard IUB nomenclature (R: A or G, Y: C or T, M: A or C, K: G or T, S: G or C, W: A or T).

Figure 2 discloses the amino acid sequence of the expression product (SEQ ID NO: 2) from the cDNA of Figure 1.

Figure 3 illustrates the relative potencies of GLP-2 peptide and GLP-1 peptides for the receptor encoded by SEQ ID NO: 1.

Figure 4 discloses a cDNA sequence of 667 nucleotides (SEQ ID NO: 9) which encodes a 222 amino acid fragment (SEQ ID NO: 10) of a human GLP-2 receptor.

Figure 5 discloses the amino acid sequence (SEQ ID NO: 10) expressed from the cDNA of Figure 4.

Figure 6 discloses a cDNA sequence (SEQ ID NO: 11), nucleotides 121-1779 of which encode a human GLP-2 receptor.

Figure 7 discloses the amino acid sequence of the expression product (SEQ ID NO: 12) from the cDNA of Figure 6.

Figure 8 illustrates the functional activation by GLP-2 peptide of the human receptor encoded by SEQ ID NO: 11 (Figure 8).

Figure 9 compares the amino acid sequences of the rat GLP-2 receptor and the human GLP-2 receptor.

Figure 10 compares the amino acid sequences of the rat GLP-2 receptor and the human GLP-2 receptor against rat GLP-1 receptor.

DETAILED DESCRIPTION AND PREFERRED EMBODIMENTS

The invention relates in one respect to polynucleotides, in their isolated form, that code for GLP-2 receptors. As used herein "isolated" means separated from polynucleotides that encode other proteins. In the context of polynucleotide libraries, for instance, GLP-2 receptor-encoding polynucleotide is considered "isolated" when it has been selected, and hence removed from association with other polynucleotides within the library. Such polynucleotides may be in the form of RNA, or in the form of DNA including cDNA, genomic DNA and synthetic DNA. The GLP-2 receptors are characterized by structural features common to the G-protein coupled receptor class, including seven transmembrane regions, and by the functional properties of binding GLP-2 peptide selectively, i.e.,

preferentially, to GLP-1 peptide. Such selective binding is revealed as a statistically significant greater binding affinity of GLP-2 than of GLP-1, in the context of the assay chosen to measure such affinity. When expressed functionally in a host cell, i.e., in operable linkage with a responsive second messenger system the GLP-2 receptors are capable further of responding to GLP-2 binding by signal transduction. In this regard, the activity of a G-protein coupled receptor such as a GLP-2 receptor can be measured using any of a variety of appropriate functional assays in which activation of the receptor results in detectable change in the level of some second messenger system, such as adenylate cyclase, calcium mobilization, inositol phospholipid hydrolysis products or guanylyl cyclase.

With reference to Figure 9 and Figure 10, which reveals homologies across amino acid sequences representing human and rat GLP-2 receptors, regions of 100% identity are indicated by solid vertical bars. In embodiments of the invention, the GLP-2 receptors are defined structurally as receptors that incorporate these regions of amino acid sequence and that also exhibit the functional characteristic of binding GLP-2 peptide selectively, relative to GLP-1 peptide. In more specific embodiments, the GLP-2 receptor structure further incorporates those amino acids which, across the human and rat receptor species, are highly conserved (indicated by ':'). At these sites, it will be appreciated that the sequence can contain any amino acid within the highly conserved amino acid family to which the identified amino acid belongs. In still more specific embodiments, the GLP-2 receptors have a structure which still further incorporates to the moderately conserved amino acids (indicated by '.') meaning, at these sites, that the amino acids within the moderately conserved family to which they belong. Beyond these sequences, the GLP-2 receptor structure can vary widely in embodiments of the invention, in allowing for non-conservative amino acid substitutions.

In one embodiment of the invention, the GLP-2 receptor is a rat GLP-2 receptor having the amino acid sequence of SEQ ID NO:2. In a specific embodiment of the invention, this rat GLP-2 receptor is encoded by the polynucleotide sequence of SEQ ID NO: 1. This particular GLP-2 receptor-encoding polynucleotide, also referred to as the WBR

gene, is a cDNA of rat origin. The expression product of this polynucleotide incorporates the mature form of the GLP-2 receptor, and additionally incorporates a secretion signal that is removed before membrane integration of the mature GLP-2 receptor product. Such a signal sequence may be naturally present on the polypeptides or replaced with a functionally equivalent secretion signal which is heterologous to the GLP-2 receptor. The replacement secretion signal chosen will depend on the expression system being used and will typically, but not essentially be endogenous to the chosen host and, also typically but not essentially, be homologous to the chosen expression controlling sequences.

The expressed rat GLP-2 receptor product (Figure 2, SEQ ID NO: 2) is characterized structurally as a single 550 amino acid polypeptide chain having a predicted molecular weight of 72kDa. Two functional translation start sites of the rat GLP-2 receptor have been identified, these are the codons encoding methionine 1 and methionine 42 of SEQ ID NO:2. Without wishing to be limited, by analogy with the GLP-1 receptor, it is believed that residues 1-66 of SEQ ID NO:2 are cleaved to provide a mature protein (i.e., the amino acid sequence of the receptor as it appears in the cell membrane) of 484 amino acids. With respect to structural domains of this GLP-2 receptor, hydropathy analysis and sequence alignment with related members of this sub-family of G protein coupled receptors indicates seven putative transmembrane domains, one spanning residues 181-203 inclusive (TM I), another spanning residues 211-230 (TM II), a third spanning residues 262-285 (TM III), a fourth spanning residues 300-321 (TM VI), a fifth spanning 339-362 (TM V), a sixth spanning 386-405 (TM VI) and a seventh spanning 422-441 (TM VII). Based on this assignment, it is likely that this GLP-2 receptor, in its natural membrane-bound form, consists of a an N-terminal extracellular domain, followed by a hydrophobic region containing seven transmembrane domains and an intracellular 442-550 amino acid C-terminal domain. The protein exhibits the highest degree of homology to the rat GLP-1 receptor with 49% identity at the amino acid level.

In a related embodiment, the GLP-2 receptor is of human origin (SEQ ID NO: 9) and incorporates the human GLP-2 receptor fragment having the amino acids of SEQ ID NO: 10.

5 This polynucleotide was isolated using the rat cDNA sequence as generally described below and as detailed in Example 3. In a further related embodiment of the invention, the cDNA is of human origin (SEQ ID NO: 11) and encodes the full length human GLP-2 receptor having residues 67-533 of the amino acid sequence of SEQ ID NO: 12. The human GLP-2 receptor precursor product (Figure 7, SEQ ID NO: 12) is characterized structurally as a single 553 amino acid polypeptide chain having a predicted molecular weight of 72 kDa. It is believed that, as for the rat GLP-2 receptor, this sequence, this precursor form of the human GLP-2 receptor incorporates an N-terminal signal sequence, which can be replaced by a functionally equivalent heterologous signal sequence. Without wishing to be limited it is believed that the mature form of the human GLP-2 receptor results after cleavage of residues 1-66 of SEQ ID NO:12 (Figure 7). With respect to structural domains of this GLP-2 receptor, hydropathy analysis and sequence alignment with related members of this sub-family of G protein coupled receptors indicates seven putative transmembrane domains, one spanning residues 181-203 inclusive (TM I), another spanning residues 211-230 (TM II), a third spanning residues 262-285 (TM III), a fourth spanning residues 300-321 (TM IV), a fifth spanning residues 339-362 (TM V), a sixth spanning residues 386-405 (TM VI), and a seventh spanning residues 422-441 (TM VII). Based on this assignment, it is likely that this GLP-2 receptor, in its natural membrane-bound form, consists of an N-terminal extracellular domain, followed by a hydrophobic region containing seven transmembrane domains interspersed with six short hydrophilic domains, and an intracellular domain, which is predicted to span residues 442-553. A second form of this GLP-2 receptor encompassed by the invention has a translational start site at the methionine codon at position 26 of the amino acid sequence presented in Figure 7 SEQ ID No. 12. The resulting 528 amino acid polypeptide chain also consists of an extracellular domain, seven transmembrane domains, and a C-terminal intracellular domain, and is at least 95% identical in sequence to residues 26-553 of the sequence presented in Figure 7, SEQ ID NO: 12.

30 In another embodiment, the invention provides GLP-2 receptor polynucleotide sequences and their unique sequence fragments as a tool useful to identify and isolate structurally related polynucleotides. At low stringency hybridization conditions, for instance, polynucleotide libraries can be probed to identify genes that are at least about 50% homologous to the GLP-

2 receptor gene. To facilitate isolation of rat GLP-2 receptor gene homologs that are also GLP-2 receptor-encoding, stringency conditions are desirably enhanced to identify homologs having at least 80% (medium stringency) sequence identity homology at the polynucleotide level to receptor gene. More desirably the WBR gene homologs are 90% identical, (high stringency) and most desirably they have at least 95% sequence (high stringency) identity when compared to WBR. Preferably, the isolated WBR homologs are characterized in that (1) they can be amplified using the PCR primers of SEQ ID NO: 3 and SEQ ID NO: 4 and (2) they bind to the probe of SEQ ID NO: 5 under high stringency conditions.

Still more preferably, the isolated homologs are those which bind, under conditions of high stringency, with consensus regions of the GLP-2 receptor-encoding polynucleotides, i.e., those regions of the rat and human GLP-2 receptor-encoding polynucleotides which are identical and, relative to GLP-1 receptor-encoding sequences, are also unique to the GLP-2 receptor-encoding polynucleotides. Alignment of this nature reveals a number consensus regions for GLP-2: for example nucleotides spanning from 1460-1786. In one embodiment of the invention, these sequences, and their complements, constitute polynucleotide fragments useful, as just described for the intact gene, to identify polynucleotides structurally related to the human and rat GLP-2 receptor embodiments of the invention.

In a related embodiment, the cDNA sequence or the unique fragments of the GLP-2 receptor, most preferably of the human GLP-2 receptor, can be used in appropriately labelled form, e.g., p^{32} labelled, for diagnosis of conditions associated with aberrant expression of the GLP-2 receptor. For example, over or under expression or expression in an inappropriate tissue. In one embodiment appropriate PCR primers (e.g., regions unique to the GLP-2 receptor but conserved between species) can be used diagnostically to identify aberrant structure or levels of GLP-2 receptor mRNA, e.g., associated with inherited or acquired disease states.

It has been found that the human GLP-2 receptor is located on chromosome 17P13. Thus, in a further embodiment, the invention provides expression products from this locus that

hybridize with the human GLP-2 receptor polynucleotide(Figure 6; SEQ ID NO:11) under stringent conditions.

As source material to isolate GLP-2 receptor-encoding homologs of the rat GLP-2 receptor-encoding polynucleotide gene, it is desirable but not necessary to use libraries of fetal or mature hypothalamal, jejunal, hindbrain or stomach tissue obtained from the vertebrate species targeted for receptor isolation. The invention accordingly includes not only the rat GLP-2 receptor-encoding polynucleotide of SEQ ID NO:1, but structural homologs thereof and particularly those that code for proteins having GLP-2 receptor properties. As exemplified hereinbelow, the WBR gene has been used successfully as a starting material to clone the human homolog of the rat GLP-2 receptor. Thus, the invention provides polynucleotides that encode GLP-2 receptors, including rat GLP-2 receptor and vertebrate homologs, particularly mammalian homologs thereof including human homologs, as well as synthetic variants of these.

It will be appreciated that such homologs can also be identified in libraries by screening, as noted, with fragments of the rat receptor gene or the human homolog, which incorporate at least 15 nucleotides, and preferably at least 25 nucleotides. With reference to SEQ ID NO: 1 and the nucleotide numbering appearing thereon, suitable nucleotide fragments include in addition to the consensus nucleotide fragments noted above, those corresponding in sequence to the extracellular GLP-2 binding domain, and the stipulated transmembrane regions and the C-terminal portion of the receptor.

Technically, the identification of GLP-2 receptor genes can be achieved by applying standard hybridization or amplification techniques to a tissue-derived polynucleotide library. A wide variety of such libraries are commercially available. Where construction of a cDNA library is necessary, established techniques are applied. For example, isolation of such a WBR homolog typically will entail extraction of total messenger RNA from a fresh source of tissue, such as hypothalamal, jejunal, stomach or hindbrain tissue, preferably hypothalamal tissue or cell lines derived from these tissues, followed by conversion of message to cDNA and formation of a library in for example a bacterial plasmid, more typically a bacteriophage.

Such bacteriophage harboring fragments of the DNA are typically grown by plating on a lawn of susceptible *E. coli* bacteria, such that individual phage plaques or colonies can be isolated. The DNA carried by the phage colony is then typically immobilized on a nitro-cellulose or nylon-based hybridization membrane, and then hybridized, under carefully controlled conditions, to a radioactively (or otherwise) labelled probe sequence to identify the particular phage colony carrying the fragment of DNA of particular interest, in this case a rat or human GLP-2 homolog. The phage carrying the particular gene of interest is then purified away from all other phages from the library, in order that the foreign gene may be more easily characterized. Typically, the gene or a portion thereof is then isolated by subcloning into a plasmidic vector for convenience, especially with respect to the full determination of its DNA sequence.

As an alternative to obtaining GLP-2 encoding DNA directly as a DNA insert from an available or a constructed cDNA library, in light of the present disclosure it can be synthesized de novo using established techniques of gene synthesis. Because of the length of the GLP-2 receptor-encoding DNAs of SEQ ID NO: 1, SEQ ID NO: 9 and SEQ ID NO: 11, application of automated synthesis may require staged gene construction, in which regions of the gene up to about 300 nucleotides in length are synthesized individually and then ligated in correct succession for final assembly. Individually synthesized gene regions can be amplified by PCR. The application of automated synthesis may typically be applied by synthesizing specific regions or fragments of the gene and ligating them, usually via designed overlaps, in correct succession to form the final gene sequence. In this case, the longer the oligonucleotide building blocks, the fewer will be the ligations needed, resulting in greater ease of assembly.

The application of automated gene synthesis techniques provides an opportunity for generating sequence variants of the naturally occurring GLP-2 receptor gene. It will be appreciated, for example, that polynucleotides coding for the GLP-2 receptor herein described can be generated by substituting one or more synonymous codons for those represented in the naturally occurring polynucleotide sequences herein provided and such "synonymous codon equivalents" are within the scope of the present invention. In addition, polynucleotides

coding for synthetic variants of the GLP-2 receptor herein provided can be generated which incorporate from 1 to 20, e.g., from 1 to 5, amino acid substitutions, or deletions or additions. Preferred sites for such a modification include areas of non homology between the rat and human sequence, for example amino acid ranges 70-92, 328-350 and 475-504. Since it will be desirable typically to retain the natural ligand binding profile of the receptor for screening purposes, it is desirable to limit amino acid substitutions, for example to the so-called conservative replacements in which amino acids of similar charge are substituted (Fig. 9), and to limit substitutions to those sites less critical for receptor activity. For example, substitution of nucleotides "G" and "A" for nucleotides "A" and "G" respectively at positions 374 and 375 of the human cDNA sequence of SEQ ID NO: 11; resulting in the replacement of the naturally occurring arginine residue at position 85 of SEQ ID NO: 12 with a glutamic acid residue, provides a functional receptor. This functional receptor is referred to herein as the Glu⁸⁵ variant human GLP-2 receptor.

Having obtained GLP-2 receptor encoding polynucleotide, GLP-2 receptor can be produced in a number of ways, including in vitro transcription and via incorporation of the DNA into a suitable expression vector and expression in the appropriate host, for example in a bacterium such as E.coli, in yeast or in insect or in a mammalian cell. A variety of gene expression systems have been adapted for use with these hosts and are now commercially available, and any one of these systems can be selected to drive expression of the GLP-2 receptor-encoding DNA. Expression vectors may be selected to provide transformed cell lines that express the receptor-encoding DNA either transiently or in a stable manner. For transient expression, host cells are typically transformed with an expression vector harboring an origin of replication functional in a mammalian cell. For stable expression, such replication origins are unnecessary, but the vectors will typically harbor a gene coding for a product that confers on the transformants a survival advantage, to enable their selection such as a gene coding for neomycin resistance in which case the transformants are plated in medium supplemented with neomycin.

These expression systems, available typically but not exclusively in the form of plasmidic vectors, incorporate expression cassettes the functional components of which include DNA

constituting expression controlling sequences and optionally also signal peptides encoding sequences, which are host-recognized and enable expression of the receptor-encoding DNA when linked 5' thereof. The systems further incorporate DNA sequences which terminate expression when linked 3' of the receptor-encoding region. Thus, for expression in the selected mammalian cell host, there is generated a recombinant DNA expression construct in which the receptor-encoding DNA is linked with expression controlling DNA sequences recognized by the host, and which include a region 5' of the receptor-encoding DNA to drive expression, and a 3' region to terminate expression.

Included among the various recombinant DNA expression systems that can be used to achieve mammalian cell expression of the receptor-encoding DNA are those that exploit promoters of viruses that infect mammalian cells, such as the promoter from the cytomegalovirus (CMV), the Rous sarcoma virus (RSV), simian virus (SV40), murine mammary tumor virus (MMTV) and others. Also useful to drive expression are promoters such as the LTR of retroviruses, insect cell promoters such as those regulated by temperature, and isolated from *Drosophila*, as well as mammalian gene promoters such as those regulated by heavy metals i.e. the metallothionein gene promoter, and other steroid-inducible promoters.

In another of its aspects, the invention provides cells or membranes derived therefrom which are adapted by genetic alteration for use, for example, in identifying GLP-2 receptor ligands. In preferred embodiments, such cells are adapted genetically by the insertion of polynucleotide coding for a GLP-2 receptor. In particularly preferred embodiments, such cells incorporate a recombinant DNA molecule, e.g. an expression construct/vector, in which DNA coding for the GLP-2 receptor and expression controlling elements functional in the host are linked operably to drive expression of the DNA. For incorporation of receptor into cell plasma membranes, the vector can, if desired, be designed to provide a suitable heterologous signal peptide sequence to substitute for the signal peptide encoded naturally within the receptor DNA.

Suitable GLP-2 producing cells include the Chinese hamster ovary (CHO) cells for example of K1 lineage (ATCC CCL 61) including the Pro5 variant (ATCC CRL 1281); the fibroblast-like cells derived from SV40-transformed African Green monkey kidney of the CV-1 lineage (ATCC CCL 70), of the COS-1 lineage (ATCC CRL 1650) and of the COS-7 lineage (ATCC CRL 1651); murine L-cells, murine 3T3 cells (ATCC CRL 1658), murine C127 cells, human embryonic kidney cells of the 293 lineage (ATCC CRL 1573), human carcinoma cells including those of the HeLa lineage (ATCC CCL 2), and neuroblastoma cells of the lines IMR-32 (ATCC CCL 127), SK-N-MC (ATCC HTB 10) and SK-N-SH (ATCC HTB 11).

For use in ligand screening assays, cell lines expressing the receptor-encoding DNA can be stored frozen for later use. Such assays may be performed either with intact cells, or with membrane preparations derived from such cells. The membrane preparations typically provide a more convenient substrate for the ligand binding experiments, and are therefore preferred as binding substrates. To prepare membrane preparations for screening purpose, i.e., ligand binding experiments, frozen intact cells are homogenized while in cold water suspension and a membrane pellet is collected after centrifugation. The pellet is then washed in cold water, and dialyzed to remove any endogenous GLP-2 receptor ligands that would otherwise compete for binding in the assays. The dialyzed membranes may then be used as such, or after storage in lyophilized form, in the ligand binding assays.

The binding of a candidate ligand to a selected GLP-2 receptor of the invention can be assessed typically using a predetermined amount of cell-derived membrane (measured for example by protein determination), generally from about 25ug to 100ug. Generally, competitive binding assays will be useful to evaluate the affinity of a test compound relative to GLP-2. This competitive binding assay is performed by incubating the membrane preparation with radiolabelled GLP-2 peptide, for example [H^3] or a radioiodinated GLP-2 analog, in the presence of unlabelled test compound added at varying concentrations. Following incubation, either displaced or bound radiolabelled GLP-2 can be recovered and measured, to determine the relative binding affinities of the test compound and GLP-2 for the

GLP-2 receptor used as substrate. In this way, the affinities of various compounds for the GLP-2 receptor can be measured.

Alternatively, binding of a candidate ligand to a GLP-2 receptor can be assessed using a functional assay. Using this approach, for example, intact cells harvested about two days following transient transfection or after about the same period following plating of stably transfected cells can be used to assess ligand binding. In a preferred embodiment, 293 EBNA cells (Invitrogen Cat. R620-07) are stably transformed with the pREP7 vector (Invitrogen Cat. V007-50) incorporating expressibly therein a GLP-2 receptor. Thereafter, binding of an agonist (or using a competition base format an antagonist) to the receptor can be discerned by measuring the level of intracellular cAMP. Most conveniently, intracellular cAMP is measured indirectly using a reporter system, wherein an easily measurable and preferably easily quantifiable downstream event indicates the level of intracellular cAMP. For example, measuring the level of the expression of a reporter gene construct having polynucleotide sequence under the control of a promoter which is responsive to cAMP. Alternatively, measurement of intracellular calcium, released from intracellular stores in response to an increase in intracellular cAMP, can be used as an indicator of the level of intracellular cAMP, for example, by incorporating into the transformed cell a protein that fluorescences on binding to calcium. In a preferred embodiment, intracellular cAMP levels are measured using the commercially available EIA kit. An additional advantage of the functionally based approach to assessing ligand binding is that the system can be automated allowing high throughput and ultra high through screening of vast chemical libraries.

As an alternative to using cells that express receptor-encoding DNA, ligand characterization may also be performed using cells for example *Xenopus* oocytes, that yield functional membrane-bound receptor following introduction of messenger RNA coding for the GLP-2 receptor. In this case, the GLP-2 receptor-encoding polynucleotide of the invention is typically subcloned into a plasmidic vector such that the introduced gene may be easily transcribed into RNA via an adjacent RNA transcription promoter supplied by the plasmidic vector, for example the T3 or T7 bacteriophage promoters. RNA is then transcribed from the inserted gene in vitro, and can then be injected into *Xenopus* oocytes. Each oocyte is a

single cell, but is large enough to be penetrated by a fine-tipped microneedle without causing irreparable damage. Following the injection of nL volumes of an RNA solution, the oocytes are left to incubate for up to several days, whereupon the oocytes are tested for the ability to respond to a particular ligand molecule supplied in a bathing solution.

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Candidate GLP-2 receptor ligands can vary widely in structure, and most suitably include proteins which are highly related to GLP-2 itself in terms of amino acid sequence. For instance, the peptides disclosed in co-pending United States patent applications WO97/39031 and WO96/32414, incorporated herein by reference, may usefully be screened for GLP-2 receptor binding activity.

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In addition to naturally occurring GLP-2 receptor sequences functional chimeric, receptors, incorporating portions of the GLP-2 receptor sequence and the polynucleotides encoding them are also embodiments of the invention. Functional chimeric GLP-2 receptors are constructed by combining the extracellular receptive sequences of a GLP-2 receptor with one or more of the transmembrane and intracellular segments of a known seven transmembrane G-protein coupled receptors for test purposes. This concept was demonstrated by Kobilka et al. (1988, Science 240:1310-1316) who created a series of chimeric $\alpha 2$ - $\beta 2$ adrenergic receptors (AR) by inserting progressively greater amounts of $\alpha 2$ -AR transmembrane sequence into $\beta 2$ -AR. The binding activity of known agonists changed as the molecule shifted from having more $\alpha 2$ than $\beta 2$ conformation, and intermediate constructs demonstrated mixed specificity. The specificity for binding antagonists, however, correlated with the source of the transmembrane domain VII. The importance of transmembrane domain VII for ligand recognition was also found in chimeras utilizing two yeast α -factor receptors and is significant because the yeast receptors are classified as miscellaneous receptors. Thus, functional role of specific domains appears to be preserved throughout the seven transmembrane G-protein coupled receptor family regardless of category.

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In parallel fashion, internal segments or cytoplasmic domains from a particular GLP-2 receptor are exchanged with the analogous domains of a known seven transmembrane G-protein coupled receptor and used to identify the structural determinants responsible for coupling the

receptors to trimeric G-proteins (Dohlman et al. (1991) *Annu Rev Biochem* 60:653-688). A chimeric receptor in which domains V, VI, and the intracellular connecting loop from β 2-AR were substituted into α 2-AR was shown to bind ligands with α 2-AR specificity, but to stimulate adenylate cyclase in the manner of β 2-AR. This demonstrates that for adrenergic-type receptors, G-protein recognition is present in domains V and VI and their connecting loop. The opposite situation was predicted and observed for a chimera in which the V->VI loop from α 1-AR replaced the corresponding domain on β 2-AR and the resulting receptor bound ligands with β 2-AR specificity and activated G-protein-mediated phosphatidylinositol turnover in the α 1-AR manner. Finally, chimeras constructed from muscarinic receptors also demonstrated that V->VI loop is the major determinant for specificity of G-protein activity.

Chimeric or modified seven transmembrane G-protein coupled receptors containing substitutions in the extracellular and transmembrane regions have shown that these portions of the receptor determine ligand binding specificity. For example, two Ser residues conserved in domain V of all adrenergic and D catecholamine receptors are necessary for potent agonist activity. These serines are believed to form hydrogen bonds with the catechol moiety of the agonists within the binding site. Similarly, an Asp residue present in domain III of all seven transmembrane G-protein coupled receptors which bind biogenic amines is believed to form an ion pair with the ligand amine group in the binding site.

Functional, cloned seven transmembrane G-protein coupled receptors are expressed in heterologous expression systems and their biological activity assessed (e.g., Marullo et al. (1988) *Proc Natl Acad Sci* 85:7551-7555; King et al. (1990) *Science* 250:121-123). One heterologous system introduces genes for a mammalian seven transmembrane G-protein coupled receptors and a mammalian G-protein into yeast cells. The seven transmembrane G-protein coupled receptor is shown to have appropriate ligand specificity and affinity and trigger appropriate biological activation--growth arrest and morphological changes--of the yeast cells.

An alternate procedure for testing chimeric receptors is based on the procedure utilizing the P_{2u} purinergic receptor (P_{2u}) as published by Erb et al. (1993, *Proc Natl Acad Sci* 90:104411-

104453). Function is easily tested in cultured K562 human leukaemia cells because these cells lack P_{2u} receptors. K562 cells are transfected with expression vectors containing either normal or chimeric P_{2u} and loaded with fura-a, fluorescent probe for Ca^{++} . Activation of properly assembled and functional P_{2u} receptors with extracellular UTP or ATP mobilizes intracellular Ca^{++} which reacts with fura-a and is measured spectrofluorometrically. As with the seven transmembrane G-protein coupled receptors above, chimeric genes are created by combining sequences for extracellular receptive segments of any newly discovered seven transmembrane G-protein coupled receptors polypeptide with the nucleotides for the transmembrane and intracellular segments of the known P_{2u} molecule. Bathing the transfected K562 cells in microwells containing appropriate ligands triggers binding and fluorescent activity defining effectors of the seven transmembrane G-protein coupled receptors molecule. Once ligand and function are established, the P_{2u} system is useful for defining antagonists or inhibitors which block binding and prevent such fluorescent reactions.

In addition to using the receptor-encoding DNA to construct cell lines useful for ligand screening, expression of the DNA can according to another aspect of the invention be performed to produce fragments of the receptor in soluble form, for structure investigation, to raise antibodies and for other experimental uses. It is expected that the extracellular portion of the GLP-2 receptor contributes significantly to the binding of ligand molecule. It is therefore desirable in the first instance to facilitate the characterization of the receptor-ligand interaction by providing this extracellular ligand-binding domain in quantity and in isolated form, i.e., free from the remainder of the receptor. Such a construct has been made for the rat GLP-1 receptor, and it was shown to bind GLP-1 (Wilmen et al. (1996) FEBS LETTS, 398:43-47).

To accomplish this, the full-length GLP-2 receptor-encoding DNA may be modified by site-directed mutagenesis, so as to introduce a translational stop codon into the extracellular N-terminal region, immediately before the sequence encoding the first transmembrane domain (TM1), i.e., before residue 181 of SEQ ID NO: 2 and before residue 181 of SEQ ID NO: 12. Since there will no longer be produced any transmembrane domain(s) to "anchor" the receptor into the membrane, expression of the modified gene will result in the secretion, in

soluble form, of only the extracellular ligand-binding domain. Standard ligand-binding assays may then be performed to ascertain the degree of binding of a candidate compound to the extracellular domain so produced. It may of course be necessary, using site-directed mutagenesis, to produce several different versions of the extracellular regions, in order to optimize the degree of ligand binding to the isolated domains.

It will be appreciated that the production of such extracellular ligand binding domains may be accomplished in a variety of host cells. Mammalian cells such as CHO cells may be used for this purpose, the expression typically being driven by an expression promoter capable of high-level expression, for example the CMV (cytomegalovirus) promoter. Alternately, non-mammalian cells, such as insect Sf 9 (*Spodoptera frugiperda*) cells may be used, with the expression typically being driven by expression promoters of the baculovirus, for example the strong, late polyhedron protein promoter. Filamentous fungal expression systems may also be used to secrete large quantities of such extracellular domains of the GLP-2 receptor.

Aspergillus nidulans, for example, with the expression being driven by the *alcA* promoter, would constitute such an acceptable system. In addition to such expression hosts, it will be further appreciated that any prokaryotic or other eukaryotic expression system capable of expressing heterologous genes or gene fragments, whether intracellularly or extracellularly would be similarly acceptable.

The availability of isolated extracellular ligand-binding domains of the receptor protein makes it feasible to determine the 3-dimensional structures of these ligand-binding regions, with or without a candidate ligand complexed thereto, by a combination of X-ray crystallographic and advanced 2D-NMR techniques. In this way, additional new candidate compounds, predicted to have the required interactions with the 3-dimensional receptor structure, can be specifically designed and tested.

With large domains, crystallography is the method of choice for structure determination of both the domain in isolation, and of the co-complex with the natural ligand (or an appropriate antagonist or agonist molecule). If a particular domain can be made small enough, for example approximately 100-130 amino acids in length, then the powerful technique of 2-D

NMR can also be applied to structure determination. This enables not only the determination of the domain structure, but also provides dynamic information about the drug-receptor interaction.

5 For use particularly in detecting the presence and/ or location, for example in intestinal tissue, the present invention also provides, in another of its aspects, labelled antibody to a GLP-2 receptor. To raise such antibodies, there may be used as immunogen either the intact, soluble receptor or an immunogenic fragment thereof, produced in a microbial or mammalian cell host as described above or by standard peptide synthesis techniques. Regions of the GLP-2
10 receptor particularly suitable for use as immunogenic fragments include those corresponding in sequence to an extracellular region of the receptor, or a portion of the extracellular region, such as peptides consisting of 10 or more amino acids of the 401-509 region of SEQ ID NO: 2. With regard to the human GLP-2 receptor (SEQ ID NO: 12), peptides comprising the mature extracellular domain (residues 65-180); intracellular loop 3 (residues 363-385) and the intracellular C-terminal domain (residues 442-533) may be usefully employed as immunogens
15 for the production of antibodies to the human GLP-2 receptor.

Antibodies to the desired GLP-2 receptor or fragment immunogen are available, for polyclonal antibody production, from the blood of an animal that has been immunized with the immunogen. Alternatively, for monoclonal antibody production, immunocytes such as splenocytes can be recovered from the immunized animal and fused, using hybridoma
20 technology, to a myeloma cells. The fusion products are then screened by culturing in a selection medium, and cells producing antibody are recovered for continuous growth, and antibody recovery. Recovered antibody can then be coupled covalently to a detectable label, such as a radiolabel, enzyme label, luminescent label or the like, using linker technology
25 established for this purpose.

Animal model systems which elucidate the physiological and behavioral roles of the GLP-2 receptor are produced by creating transgenic animals in which the activity of the
30 GLP-2 receptor is either increased or decreased, or the amino acid sequence of the expressed GLP-2 receptor is altered, by a variety of techniques. Examples of these

techniques include, but are not limited to: 1) Insertion of normal or mutant versions of DNA encoding a GLP-2 receptor, by microinjection, electroporation, retroviral transfection or other means well known to those skilled in the art, into appropriate fertilized embryos in order to produce a transgenic animal or 2) Homologous recombination of mutant or normal, human or animal versions of these genes with the native gene locus in transgenic animals to alter the regulation of expression or the structure of these GLP-2 receptor sequences. The technique of homologous recombination is well known in the art. It replaces the native gene with the inserted gene and so is useful for producing an animal that cannot express native GLP-2 receptors but does express, for example, an inserted mutant GLP-2 receptor, which has replaced the native GLP-2 receptor in the animal's genome by recombination, resulting in under expression of the transporter. Microinjection adds genes to the genome, but does not remove them, and so is useful for producing an animal which expresses its own and added GLP-2 receptors, resulting in over expression of the GLP-2 receptors.

One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are dissected out of their oviducts. The eggs are stored in an appropriate medium such as M2 medium. DNA or cDNA encoding a GLP-2 receptor is cesium chloride purified from a vector by methods well known in the art. Inducible promoters may be fused with the coding region of the DNA to provide an experimental means to regulate expression of the transgene. Alternatively or in addition, tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the trans-gene. The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a piper puller) and the egg to be injected is put in a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse (a mouse stimulated by the appropriate hormones to maintain pregnancy but which is not actually pregnant), where it proceeds to the uterus, implants, and develops to term. As noted above, microinjection is not the only methods for inserting DNA into the egg cell, and is used here only for exemplary purposes.

The invention having been described above, may be better understood by referring to the following examples. The following examples are offered for the purpose of illustrating the invention and should not be interpreted as a limitation of the invention.

EXAMPLE 1

Isolation of the GLP-2 receptor

PCR-assisted cloning of partial rat and mouse GLP-2 receptor cDNAs

Rat Neonate Intestine cDNA library (Stratagene, La Jolla, CA; Cat. 936508) and Mouse Jejunum first strand cDNA was prepared. Degenerate primers M-2F/S (SEQ ID NO: 3) and M-7R/S (SEQ ID NO: 4) were used to amplify a partial fragment of the rat GLP-2 receptor from the Rat Neonate Intestine cDNA library and of the mouse GLP-2 receptor from Mouse Jejunum template. The protocol is described below:

Degenerate PCR :

6 µl 10x VENT buffer from New England Biolabs

6 µl 2.5µM each stock dATP, dCTP, dGTP and dTTP

4 µl rat neonate intestine cDNA (1:10 dilution)

3 µl 25µM M2F/S primer [5'-TTTTTCTAGAASRTSATSTACACNGTSGGCTAC-3']
(SEQ ID NO: 3)

3 µl 25µM M7R/S primer [5'-TTTTCTCGAGCCARCCASSWRTARTTGGC-3']
(SEQ ID NO: 4)

2 µl (10 units) Amplitaq DNA polymerase (Perkin Elmer)

36 µl ddH₂O.

Reaction conditions: 35 cycles at 94°C, 2°min.; 94°C, 1 min.; 53°C, 30 sec.; 72°C, 1 min.

The predominant PCR product was a 303 base pair (bp) DNA fragment. 30 µl samples of the above PCR were purified using the QIAGEN PCR purification kit and eluted in 30 µl ddH₂O. The resulting product was then re-amplified using the same degenerate PCR conditions, with the exception only of 31 cycles at 94°C.

The predominant product at 303 base pair (bp) was cut out and purified using QIAGEN QIAquick gel purification protocol into 30 µl ddH₂O. The resulting product was then re-amplified using the same degenerate PCR conditions, with the exception only of 31 cycles at 94°C.

Next, double digest (Xba I and Xho I) was done on the entire reamplified PCR reaction as follows: 28 µl DNA; 16 µl 10X One-Phor-All buffer (Pharmacia); 2 µl (40 units) Xba I enzyme (Pharmacia); 2 µl (40 units) Xho I enzyme (Pharmacia); and 30 µl ddH₂O.

The samples were digested 4 hours in 37°C water block heater, brought up to 100 µl volume with ddH₂O (sterile) and purified by (1) equal amount (100ul) chloroform extraction; (2) weekend precipitation with 2 volumes ethanol/10 volumes 3M sodium acetate; (3) 1x wash with 70% EtOH; and (4) resuspension in 10 µl 1x TE (pH 8.0).

pBluescript clone 5HT1F#9 was next digested with Xba I and Xho I as follows:

10 µl DNA (pBluescript clone 5HT1F#9)

5 µl 10X NEBuffer 2 (New England Biolabs)

3 µl (1:20 dilution=3 units) Xba I (New England Biolabs)

3 µl (1:20 dilution=3 units) Xho I (New England Biolabs)

5 µl (10x) BSA (New England Biolabs)

24 µl ddH₂O.

The sample was digested for 3 hours in 37 C water block heater, heat-inactivated at 65°C for 20 min and purified using GeneCleanII kit from BIO 101. Aliquots of the PCR reactions were cloned into the above pBluescript plasmid vector using T4 DNA ligase kit (New England Biolabs) and transformed into Epicurean Coli XL-2 Blue MRF' Ultracompetent cells (Stratagene). The transformation was plated onto 2xYT + AMP plates and single colonies were picked. DNA minipreps were made using QIAGEN QIA-prep 8 miniprep kit and the sequences of the fragments were determined using ABI system. Novel sequences were identified containing a partial fragment of the rat and mouse GLP-2 receptor sequence.

Cloning of cDNA with complete GLP-2 receptor coding region was achieved as follows:
First, cDNA libraries from the following three tissues were used for screening,

1. Rat Hypothalamus (RHT)
2. Rat Hind Brain (RHB)
3. Rat Duodenum and Jejunum (RDJ)

The three cDNA libraries were prepared by priming with random primer and subcloning unidirectionally into Hind III and Not I sites of pcDNA3.

Next, the three cDNA libraries were homology screened by a degenerate oligo C4-4 [5'-AACTACATCCACMKGMAYCTGTTYVYGTCBTTCATSCT-3'] (SEQ ID NO: 5) by colony lifts and filter hybridization. The following hybridization conditions were employed: 5X SSPE (1X SSPE is 0.18M NaCl, 10mM NaH₂PO₄ (pH 7.4), 10mM EDTA (pH 7.4)) and 5X Denharts solution (1% Ficoll, 1% Polyvinylpyrrolidone, 1% BSA); 25 mg/ml salmon sperm DNA.

Filters were hybridized at 50°C overnight. Then the filters were washed 2 times in 2X SSPE and 1% SDS at room temperature for 30 min, 2 times in 2X SSPE and 1% SDS at 50°C for 20 min per wash, and finally two times in 1X SSPE and 0.5% SDS. Positive clones were identified by autoradiography. A plug of 1cm² surrounding the positive clone was removed from the plate and placed in 1 ml of 2x YT +20% Glycerol, vortexed and was frozen at -80°C.

Plasmid DNA from positive plugs was prepared as follows: 100 ml of bacterial culture of each positive plug was grown on an agar plate. The bacterial cells were scraped and resuspended in 1 ml of 2xYT medium+20% Glycerol. Bacterial pellet from the 250 ml of bacterial resuspension was resuspended in 150 ml of Solution I (50mM Glucose, 10mM Tris-HCl, 1mM EDTA), lyse in Solution II (0.2M NaOH, 1% SDS), neutralized with ice cold Solution III (Potassium acetate; 4 vol.of 5M potassium acetate + 1 vol. of 10M acetic acid). After pelleting bacterial DNA, 340 ml isopropanol was added to the supernatant. This was centrifuged at max for 15 min. The pellet was resuspended in TE + 20 mg/ml RNase, incubated at 37 C for 30 minutes and precipitated with isopropanol

+ 0.2M potassium acetate. After centrifugation, the pellet was washed with 70% alcohol, allowed to air dry and resuspended in TE.

5 Plasmid DNA from 2777-clone pools of rat hypothalamus cDNA library RHT cDNA library was next exploited as follows: Two primers were designed from an area of the PCR-cloned GLP-2 receptor cDNA sequence that did not have identity to known receptors of the gene family. The two primers P23-R1 and P23-F1 amplified a 196 bp fragment only from novel clone DNA but not with GLP-1 receptor cDNA or PACAP receptor cDNA. The Expand™ PCR system from Boehringer Mannheim (Cat. 1681-842) was used under the following conditions:

10 2 µl of 10x Expand™ Buffer 1

2.8 µl of 2.5mM dNTP mix

0.6 µl of primer P23-R1 [5'-TCATCTCCCTCTTCTTGGCTCTTAC-3'] (SEQ ID NO: 6)

0.6 µl of primer P23-F1 [5'-TCTGACAGATATGACATCCATCCAC-3'] (SEQ ID NO: 7)

0.3 µl of Expand PCR enzyme (1 unit)

12.7 µl water

1 µl DNA

Reaction conditions: 32 cycles at 93°C, 40 sec; (cycles) 58°C, 40 sec; (cycles) 68°C, 40 sec

DNAs from each positive plug or pool of 2777-clone pools were amplified with specific primers P23-F1 and P23-R1 under the conditions specified above. Out of 1057 C4-4 hybridization-positive plugs and 884 2777-clone pools only five template sources amplified a 196 bp PCR product. These were: (1) Plug 334, (2) Plug 780, (3) RHT pool 233, (4) RHT pool 440, and (5) RHT pool 587.

Amplification of GLP-2R cDNA from the five positive templates was then performed. By using one specific primer (P23-R1 or P23-F1) and one primer based on pcDNA3 vector (Invitrogen) sequence (830F or 1186R), the GLP-2R cDNA insert was directly

amplified from clonally impure plugs or 2777-clone pools. The sequences of the vector primers were as follows:

830F: [5'-AACCCACTGCTTAC-3']

1186R: [5'-CCCAGAATAGAATGACACC-3']

The PCR was done, under the following conditions just noted, using Expand™ PCR system.

The most prominent band was reamplified, purified and sequenced. Based on the amplified sequence obtained, additional primers were designed and new sequencing carried out. In this manner the complete sequences of the GLP-2R cDNA inserts in all five sources of clones were determined. Sequence analysis showed that only pool RHT 440 and pool RHT 587 contain clones with complete coding sequence of GLP-2R and that the two clones were identical (derived from the same cDNA clone).

Because of difficulty in clonally purifying the GLP-2 receptor cDNA clone from the RHT 440 or RHT 587 cDNA library pools, the cDNA was amplified and recloned into pcDNA3. Based on the sequence obtained from RHT 440 and RHT 587, two primers were designed one which primed starting 4bp upstream of the initiation codon and another which primed starting 8bp downstream of the stop codon.

WBR-C5: [5'-CAGGGGCCGGTACCTCTCCACTCC-3']

WBR-C3: [5'-TTGGGTCCTCGAGTGGCCAAGCTGCG-3']

The two primers were used to amplify a DNA fragment of approximately 1525 bp fragment under the following PCR conditions using Expand™ PCR system from Boehringer Mannheim (Catalogue no. 1681-842).

10 µl of 10x Expand™ PCR Buffer 1

14 µl of 2.5mM dNTP mix

3.0 µl of Primer 1 (10 µM) (WBR-C5)

3.0 µl of Primer 2 (10 µM) (WBR-C3)

1.5 µl of Enzyme (5 units)

63.5 µl water

5 µl DNA

Reaction conditions: 5 cycles (93°C, 1 Min; 72°C, 40s; 60°C, 45 sec; 68°C, 2 min) and 25 cycles (93°C, 1 min; 72°C, 1 min; 68°C, 2 min).

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The amplified product was subcloned into Kpn I and Xho I sites of pcDNA3 vector (Invitrogen). Plasmid DNA was prepared using the method described above.

EXAMPLE 2

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Functional Assay

Cos-1 cells were transfected as described in Analytical Biochemistry, 218:460-463(1994) with Rat clone 587 GLP-2 receptor, cloned human GLP-2 receptor (pC3/HuGL2R-2), or cloned residue 85 variant human GLP-2 receptor (pC3.1/HuGL2R-MH4), pcDNA3. Rat GLP-1 (7-36) amide was used as a control peptide. Solutions used were as follows:

RSC in RPMI 1640 (49ml RPMI + 1ml FCS + 50ul chloroquine, 100mM);

DEAE/RSC Solution: 18.4 ml RSC +1.6 ml DEAE/Dextran (10 mg/ml).

The assay procedure entailed the following:

a) 50 mg of either rat clone 587 GLP-2 receptor, or cloned human GLP-2 receptor, or cloned residue 85 variant human GLP-2 receptor was added (as plasmid pcDNA3) to a 50 ml tube containing six mls. of RSC and incubated at 37°C.

b) Six ml of DEAE/RSC solution was added to each tube and incubated at 37°C for 2 min.

c) 1.5 ml of COS-1 cell suspension (5.5 millions cells) was added to each tube and incubated for 1 hr 45 min at 37°C.

d) Following incubation, the sample was spun for 5 minutes at low speed, washed with DMEM/F12 + 10% FBS twice, and the pellet resuspended in 12.5 ml DMEM/F12 + 10% FBS media.

e) One ml of cell suspension (step d) was added to each well of 6 well plates coated with poly- D-lysine (from Collaborative Biomedical), containing 3 ml of media (0.45 million cells/well).

f) Plates were incubated at 37°C for 3 days.

Treatment of Transfected Cos-1 cells with GLP-1/GLP-2 analog was done as follows:

Solutions : DMEM/F12 (SFM)+ IBMX (3-isobutyl-1-methylxanthin) 0.85mM +0.1% ascorbic acid and 10 um pargyline (all solutions purchased from Sigma). Media was prepared fresh on day of use.

Assay Procedure: The culture media of each well (transfected 6 well plates, cells) was removed, and the wells were washed once with SFM media. Then 2 ml of SFM + IBMX media was added to each well and plates were incubated at 37°C for 10 min.

Following incubation, the SFM + IBMX was removed from each well and fresh SFM + IBMX media containing GLP-1/GLP-2 (GLP-1,7-36,amide from Sigma, [Gly2]hGLP-2 from Allelix) concentration 1, 3, 10 and 30 nM were added to the appropriate wells.

Plates incubated at 37°C incubator for 30 min. Following incubation, the media were removed from each well. The wells were washed once with 1ml PBS (Phosphate Buffered Saline). Each well was then treated with 1ml cold 95% ethanol: 5mM EDTA (2:1) at 4°C for 1 hr. Cells from each well then were scraped and transferred into individual eppendorf tubes. Tubes were centrifuged for 5 min at 4°C, and the supernatants were transferred to new eppendorf tubes and dried in speed vacuum.

Following drying, tubes were reconstituted in 100ul of Na- Acetate and kept at 4°C, 25 µl of this solution used for cAMP Assay.

The functional assay was performed as follows: cAMP content for each extract was determined in duplicate by EIA (Enzyme ImmunoAssay) using the Amersham Biotrak cAMP EIA Kit (Amersham 225). Results of the assays, illustrated in Figure 3 and Figure 8, demonstrate the GLP-2 selectivity exhibited by the cloned rat and human receptors. In a similar functional assay used to users binding to the GLP-1 receptor, the expected specificity for GLP-1 was observed.

EXAMPLE 3

Isolation Of Human GLP-2 Receptor cDNA

Medium-stringency hybridization screening of a human hypothalamus cDNA library

One million clones from a λ gt10 cDNA library from human hypothalamus (Clontech; Cat. No. 1172a) were screened by plaque lifts on nitrocellulose filters (Amersham; Cat.RPN137E). The probe was prepared by random primer labelling of a DNA fragment containing the complete coding region of rat GLP-2 receptor. The DNA fragment was isolated from clone 587-C1, which contains the complete coding region from SEQ ID NO: 2.

Pre-hybridization and hybridization were each carried out overnight in a hybridization solution consisting of 50% formamide, 5X SSPE, 5X Denhart's solution, 0.5% SDS and salmon sperm DNA (200 mg/ml). After hybridization the filters were washed under the following conditions (medium stringency):

two times at room temperature in 2X SSPE and 0.01% SDS.

two times at 42°C in 2X SSPE and 0.01% SDS.

two times at 42°C in 0.2X SSPE and 0.01% SDS.

The filters were autoradiographed and agar plugs, each containing numerous bacteriophage plaques, were picked from regions on the plates corresponding to positive signals on the filter. From one million cDNA clones sampled in the first round screen, there were identified two positive clones (HHP6-1 and HHP13). On secondary screening only HHP13 turned out positive. Several positive plaques (HHS13) from the HHP13 plate were pooled and taken for tertiary screening. Three single positive plaques from this round of screening were picked (HHT13-1, HHT13-2, HHT13-3).

PCR amplification was then used for partial sequencing of the positive clones. On a lawn of bacterial cells (E.coli C600Hfl), 10 μ l of phage suspension from each clone was applied at marked spots. After 5 hr incubation at 37°C, the phage plaques were clearly visible and covered ~ 1 cm². A portion of each plaque was transferred to 200 μ l of water. The samples were incubated in a boiling water bath for 5 min and centrifuged at room temperature for 10 min. One millilitre of sample was used for PCR amplification with two sets of degenerate primers:

M2FS[5'-TTTTTCTAGAASRTSATSTACACNGTSGGCTAC-3'] (SEQ ID NO: 3) and

M7RS[5'-TTTCTCGAGCCARCARCCASSWRTARTTGGC-3'] (SEQ ID NO: 4); or
C4-4 [5'-AACTACATCCACMKGMAYCTGTTYVYGTCBTTTCATSCT-3'] (SEQ ID
NO: 5) and

C9-2R [5'-TCYRNCTGSACCTCMYYRTTGASRAARCAGTA-3'] (SEQ ID NO: 8).

The ExpandTM PCR system from Boehringer Mannheim (Cat. 1681-842) was used under
the following conditions:

5 μ l of 10x ExpandTM Buffer 3

7 μ l of 2.5mM dNTP mix

1.5 μ l of primer M2FS or C4-4

1.5 μ l of primer M7RS (with M2FS) or C9-2R (with C4-4)

0.75 μ l of Expand PCR enzyme (1 unit)

33.25 μ l water and

1 μ l DNA.

Reaction conditions were: 32 cycles at 93°C, 1 min; cycles 50°C, 1 min; cycles 45°C, 1
min; cycles 68°C, 2 min.

M2F/S and M7R/S amplified a DNA fragment of about 300 bp and C4-4 and C92-R
amplified a DNA fragment of about 700 bp. The PCR products were purified using the
QIAGEN QIAquick PCR purification kit (Cat. 28104) and eluted in 50 μ l 10 mM Tris,
pH 8.0. Sequence analysis of the products revealed no differences between the
templates, as expected from the fact that they represent multiple copies of a single cDNA
clone (HHT13).

A number of factors indicate that this clone contains coding sequence of the human
GLP-2 receptor. One factor is the degree of sequence similarity. The glucagon receptor
cDNA can be used to predict the expected degree of sequence conservation found
between rat and human receptors. At the nucleotide level, there is 82.6% identity within
the coding regions of the rat and human glucagon receptors. At the amino acid level,
there is 80.9% identity and 89.1% amino acid similarity between the glucagon receptors
of the two species.

In the case of the human GLP-2 receptor cloned herein, the sequence of the partial human GLP-2 receptor cDNA (HHT13) is highly homologous to rat GLP-2 receptor cDNA at both the nucleotide and amino acid level. SEQ ID NO: 9 shows 87.1% identity with the rat GLP-2 receptor cDNA sequence. The predicted amino acid sequence of this cDNA region has 87.4% identity and 93.2% similarity with the predicted amino acid sequence of the rat GLP-2 receptor. The total predicted length of the rat receptor preprotein is 550 amino acids, suggesting about 44% of the coding region of the human receptor had been identified.

Further evidence supporting this conclusion comes from a comparison of the partial human GLP-2 receptor amino acid sequence with the rat GLP-2 receptor and the 3 next closest family members, shown below:

Receptor Sequence (amino acid)	Percent Identity with HHT13	Percent Similarity
GLP-2 receptor (rat)	87.4	93.2
GLP-1 receptor (rat)	50.0	74.1
Glucagon receptor (rat)	51.4	73.9
GIP receptor (rat)	50.7	70.3

These comparisons, together with the benchmark provided by sequence similarities between the rat and human glucagon receptors, provide definitive evidence that the cDNA designated HHT13 represents a fragment of the human counterpart of the rat GLP-2 receptor.

The full amino acid sequence of the human GLP-2 receptor can be obtained by first determining the sequence of the complete cDNA inserts in HHT13-1, HHT13-2 and HHT13-3. By using degenerate primers for PCR amplification and subsequent sequencing, we obtained sequence from only part of each insert. It is possible that these identical clones contain an insert which spans the complete coding sequence of the human GLP-2 receptor preprotein. To determine the complete sequence of the cDNA insert, the clones are grown in large quantity to prepare approximately 20 mg of each

equivalent clone. The complete cDNA insert is excised by restriction with Eco RI, and subcloned into pcDNA3 (Invitrogen). Alternatively, two primers from vector sequence flanking the insert are used to amplify the complete cDNA insert using the ExpandTM PCR system from Boehringer Mannheim (Cat. 1681-842). The amplified cDNA is cut with appropriate restriction enzymes and is subcloned into pcDNA3 (Invitrogen).

If a complete coding sequence is not present in the HHT13 clones, cDNA libraries are screened for additional clones to complete the coding region of human GLP-2 receptor cDNA. Preferably human cDNA libraries (from Stratagene or Clontech) representing the following tissues are used for screening: Human hypothalamus; Human fetal brain; Human duodenum and jejunum; Human stomach; and Human fetal intestine.

Two PCR primers are designed from the sequence of human GLP-2 receptor cDNA already determined. These primers are designed such that they could not amplify any related gene family members other than the GLP-2 receptor cDNA itself. A dilution of the cDNA library stock is used to make library sub-pools such that 50,000 clones are represented in each pool. PCR is conducted with the GLP-2 receptor-specific primers to diagnose pools containing a GLP-2 receptor cDNA clone, using the ExpandTM PCR system from Boehringer Mannheim (Cat. 1681-842) under the following conditions:

2 µl of 10x ExpandTM Buffer 1

2.8 µl of 2.5mM dNTP mix

0.6 µl of primer P1

0.6 µl of primer P2

0.3 µl of Expand PCR enzyme (1 unit)

12.7 µl water

1 µl of library pool containing 50,000 clones

Reaction conditions: 32 cycles at 93°C, 40 sec; 50-58°C, 40 sec; 68°C, 40 sec.

Sequence is then obtained from the complete GLP-2 receptor cDNA insert from a positive pool. By using one specific primer and one primer based on vector sequence close to the cloning site, the GLP-2 receptor cDNA insert is directly amplified from

clonally impure clone pools, using the Expand™ PCR system from Boehringer Mannheim (Catalogue no. 1681-842) most suitably under the following conditions:

2 µl of 10x Expand™ Buffer 1

2.8 µl of 2.5mM dNTP mix

0.6 µl of Primer 1

0.6 µl of Primer 2

0.3 µl of Enzyme (1 unit)

12.7 µl water

1 µl of library pool stock

Reaction conditions: 32 cycles at 93°C, 45 sec; 50°C, 45 sec; 68°C, 1 min.

The reaction is run on a preparative agarose gel, and the most prominent band is purified and sequenced. Based on the amplified sequence obtained, additional primers are designed to obtain sequence and clones of complete coding region and clone the complete cDNA

5' RACE and 3' RACE are used to obtain complete coding sequence of the human GLP-2 receptor cDNA. Rapid Amplification of cDNA Ends (RACE) is a procedure routinely used for amplification of DNA sequences from first cDNA strand (easily prepared from mRNA) template between a defined internal site and either 3' or the 5' end of the mRNA. Total or mRNA from different human tissues are commercially available from Clontech. The 3' RACE System (Gibco-BRL Life Technologies; Cat. 18373-019) and 5' RACE System (Cat. 18374-058) kits are used. The manuals of these two products provide detailed protocols. In brief, protocols are as described below.

For the 3' RACE procedure, first strand cDNA synthesis is initiated at the poly (A) tail of mRNA using the adapter primer (provided with system) incorporating a unique sequence for universal PCR amplification of the RACE products. After synthesis of the first strand cDNA from this primer, the original mRNA template is destroyed with RNase H. Amplification is then performed using two primers: one is a gene-specific primer (which will be designed from the available partial cDNA sequence of HHT13);

the other is the universal amplification primer provided with the kit. The amplified product is subcloned into a plasmid vector for sequencing.

For the 5' RACE System, the first strand cDNA is synthesized from mRNA using a gene-specific primer (which is based on the available partial cDNA sequence of HHT13) and SuperScript II reverse transcriptase. The original mRNA template is removed by treatment with RNase H. Unincorporated dNTPs, primer, and proteins are separated from cDNA using spin cartridge. A homopolymeric dCTP tail is then added to the 3'-end of the first strand cDNA using TdT enzyme and dCTP nucleotides. PCR amplification is performed using two primers: one is a nested, gene-specific primer designed from the available partial DNA sequence of HHT13; and the other is an "anchor primer" provided with the system. Both primers incorporate restriction sites for subcloning into plasmids and subsequent sequencing.

Sub-cloning of HHT13 λ gt10 clones into pcDNA3, their sequencing and expression

A. Amplification of cDNA inserts with λ gt10 primers.

On a lawn of bacterial cells (E.coli C600Hfl), 10 μ l of phage resuspension from each clone was placed at marked spots. After 5 hr incubation at 37°C, the phage plaques were clearly visible. The surface of each plaque was transferred to 200 μ l of water. The samples were kept in boiling water bath for 5 minutes and centrifuged at room temperature for 10 minutes. 1 μ l of sample was used to amplify with a set of λ gt10 primers.

GT10-5KXb [5'-GGGTAGTCGGTACCTCTAGAGCAAGTTCAGCC-3']

vs

GT10-3BXh [5'-ATAACAGAGGATCCTCGAGTATTTCTTCCAG-3']

The Expand™ PCR system from Boehringer Mannheim (Cat. 1681-842) was used under the following conditions:

5 μ l of 10x Expand™ Buffer 3

7 μ l of 2.5mM dNTP mix

1.5 µl of primer GT10-5KXb
1.5 µl of primer GT10-3BXh
0.75 µl of Expand PCR enzyme (1 unit)
33.25 µl water
5 1 µl DNA

Reaction conditions: 5 cycles of 93°C-40 sec; 50 °C- 1 min; 68°C-2 min and 30 cycles
of 93°C-40 sec; Ramp to 68°C- 1 min; 68°C-2 min.

10 An amplified DNA fragment of about 2200 bp long was seen on the agarose gel from all
three clones. The PCR product were purified using the QIAGEN's QIAquick PCR
purification kit (Cat. no. 28104) and eluted in 50 µl 10 mM Tris, pH 8.0. The templates
were sequenced.

B. Subcloning into pcDNA3 vector.

20 The amplified and purified DNA from the three clones was restricted with Kpn I and
Xho I and subcloned into pcDNA3 restricted with similar restriction enzymes. The
plasmids were named pHHT13-1, pHHT13-2, and pHHT13-3. Plasmids DNAs were
prepared using either crude method (alkaline treatment, bacterial DNA precipitation with
3 M KOAc, isopropanol precipitation followed by RNase treatment and second round of
isopropanol precipitation) or plasmid DNA kits from Qiagen Inc. The templates
prepared using Qiagen's kits were sequenced.

C. Functional Assay

25 Transfections were carried out with each clone, using the rat GLP-2R, 587 clone as a
positive control for cAMP response to GLP-2 peptide. Methods for transfection, cell
culture and cAMP assay were identical to those described for the functional assay of rat,
587 clone. Results showed that although the positive control gave good cAMP response
in COS cells, none of the HHT13 clones gave any cAMP response. As confirmed by
sequencing which showed a frame-shift mutation, the functional data suggested that no
30 functional GLP-2R protein was expressed from these cDNA clones.

D. Comparison of DNA sequences between rat GLP-2R and HHT13 subclones.

The comparison showed a 2 bp deletion at a position corresponding to nucleotides 389-390 of the rat GLP-2R cDNA, resulting in the loss of nucleotides 374-375 of the human GLP-2R cDNA sequence presented herein.

PCR was used to incorporate two bp of the rat GLP-2R DNA sequence into HHT13-1 DNA at the site of the 2 bp frame-shift deletion identified relative to the rat GLP-2R coding sequence. The following primers were designed from HHT13 DNA sequence to insert two bp :

HWBR/2BPI-475F

[5'-ACAGGCATGTCTGGAAGACTTACTCAAGGAACCTTCTGGCAT-3']

HWBR/2BPI-506R

[5'-ATGCCAGAAGGTTCTTGAGTAAGTCTTCCAGACATGCCTGT-3']

HWBR-F7 [5'-TTCCTCTGTGGTACCAAGAGGAATGC-3']

and HWBR-1910R:

[5'-GGTGGACTCGAGGTACCGATCTCACTCTCTTCCAGAATC-3']

PCR 1: One ng of pHHT13-1 DNA was used as template to do two PCRs with primers, HWBR-F7 vs HWBR/2BPI-506R and HWBR/2BPI-475F vs HWBR-1910R. The Expand™ PCR system from Boehringer Mannheim (Cat. 1681-842) was used under the following conditions:

5 µl of 10x Expand™ Buffer 1

7 µl of 2.5mM dNTP mix

1.5 µl of primer HWBR-F7 or HWBR/2BPI-475F

1.5 µl of primer HWBR/2BPI-506R or HWBR-1910R

0.75 µl of Expand PCR enzyme (1 unit)

33.25 µl water and

1 µl DNA.

Reaction conditions: 10 cycles of 92°C-40 sec; 48°C- 1 min; 68°C-3 min and 30 cycles of 92°C-40 sec; 55°C- 40 sec; 68°C-2 min.

The primers HWBR-F7 and HWBR/2BPI-506R amplified a DNA fragment of 400 bp and HWBR/2BPI-475F and HWBR-1910R amplified a DNA fragment of approximately 1.4 kb on an agarose gel. The two bands were cut out of the agarose gel and purified with Qiaquick gel extraction kit from Qiagen Inc. (Cat no. 28706) and the DNAs were eluted in 50 µl of 10 mM Tris (pH 8.5).

PCR 2 (Extension without primers): Approximately 75 ng of two amplified product from above PCR 1 were mixed and then recombined without primers by extending under the following conditions:

2 µl of 10x Expand™ Buffer 1

2.8 µl of 2.5mM dNTP mix

0.3 µl of Expand PCR enzyme (1 unit)

8.9 µl water

6 µl of combined PCR 1 products

Reaction conditions: 15 cycles of 92°C-1 min; 60°C- 5 min; 68°C-3 min.

PCR 3: 1 µl of amplified mix from PCR 2 was used as template to amplify with HWBR-F7 and HWBR- 1910R primers using the following conditions:

10 µl of 10x Expand™ Buffer 1

14 µl of 2.5mM dNTP mix

3.0 µl of primer HWBR-F7 or HWBR/2BPI-475F

3.0 µl of primer HWBR/2BPI-506R or HWBR-1910R

1.5 µl of Expand PCR enzyme (1 unit)

67.5 µl water and

1 µl DNA.

Reaction conditions: 30 cycles of 92°C-1 min; 60°C- 1 min; 68°C-2 min.

A DNA fragment of approximately 1.7 kb was amplified as seen on an agarose gel. The PCR product was purified using the QIAGEN's QIAquick PCR purification kit (cat. no. 28104) and eluted in 50 µl of 10 mM Tris, pH 8.0. The purified product was restricted with Kpn I and subcloned into Kpn I-restricted pcDNA3.1(-)/Myc-His A (Invitrogen,

Cat. no. V855-20). One clone, named pc3.1/HuGL2R/MH6 (pHuMH6), had the 1.7 kb insert in correct orientation as checked by PCR using vector vs. insert primers.

Functional assay:

This hybrid clone was compared to rat GLP-2R using the assay described in Example 2. Results showed that the 2 bp "GA" replacement into the putative deletion site yielded a clone encoding a functional GLP-2R protein, as shown by the cAMP response to GLP-2 treatment.

EXAMPLE 6

Isolation of the Full-Length Human GLP-2 Receptor cDNA

Twenty thousand clones from λ gt10 cDNA Library from Human Stomach (Clontech; Cat. HL3017a) were plated on each of 100 agar 150 mm plates. SM buffer (0.1 M NaCl, 10 mM Mg_2SO_4 , 35 mM Tris, pH-7.5, 0.01% gelatin) was added to each plate to obtain 100 phage lysates each containing 20,000 (20K) pooled clones. The first fifty 20K phage lysates (20K pools) were screened by PCR using two primers designed from HHT13 DNA sequence. The template DNA from each pool was prepared by boiling phage lysate for 10 minutes and centrifuging for 10 minutes.

HWBR-113F [5'-GTGGAGAGGATTTGTGCAAACATTTC-3']

HWBR-578R [5'-AGAGACATTTCCAGGAGAAGAATGAG-3']

1 μ l of each 20K pool DNA was diagnosed by PCR with HWBR-113F and HWBR-578R primers using the following conditions:

2 μ l of 10x ExpandTM Buffer 1

2.8 μ l of 2.5mM dNTP mix

0.6 μ l of primer HWBR-113F

0.6 μ l of primer HWBR-578R

0.3 μ l of Expand PCR enzyme (1 unit)

12.7 μ l water

1 μ l 20K pool DNA

Reaction conditions: 35 cycles of 92°C- 40s.; 60°C- 40s.; 68°C-1 min.

A DNA fragment of approximately 450 bp was seen in amplification of templates from two pools (HST 19 and HST 38).

B. Screening of clones from two positive pools: HST 19 and HST 38.

40,000 clones plated from each of two positive 20K pools were screened by plaque lifts on nitrocellulose filters (Amersham; Cat.RPN137E). The probe was prepared by random primer labelling a DNA fragment from pHHT13-1.

1. The filters were pre-hybridized and hybridized at 42°C overnight. Hybridization solution consisted of 50% formamide, 5X SSPE, 5X Denhart's solution, 0.5% SDS and salmon sperm DNA (200 mg/ml).

2. After hybridization the filters were washed under the following conditions:

- two times at room temperature in 2X SSPE and 0.01% SDS;
- two times at 42°C in 2X SSPE and 0.01% SDS; and
- two times at 50°C in 0.1X SSPE and 0.01% SDS.

3. The filters were autoradiographed and the regions on the plates matching to positive signals were isolated. One positive clone (HST 38-4-30) was isolated from HST 38 pool. 450 bp DNA fragment was amplified from the positive clone by using primers HWBR-113F and HWBR-578R and sequenced. The sequence clearly showed that the plasmid contain 2 bp (AG) at position 373-374 of HHT13 DNA sequence.

The complete insert of clone HST 38-4-30 was amplified using λ gt10 primers as described in Example 1. PCR amplified a DNA fragment of approximately 1.4 kb. The amplified DNA was purified and sequenced.

EXAMPLE 7

Reconstruction of a clone of full-length functional human GLP-2R cDNA and functional assay

A 700 bp fragment obtained by Kpn I and Pvu II restriction digest of the amplified DNA from clone HST 38-4-30, and 1.4 kb DNA fragment from Xho I and Pvu II restricted pHHT13-1 DNA were subcloned into Kpn I and Xho I restricted pcDNA3 in a three-way ligation. The new plasmid construct was called pc3/HuGL2R-2. In this manner the full length sequence of the human GLP-2 receptor was obtained.

Functional Assay:

The new clone was compared to the rat GLP-2R clone 587 as described previously above. Results showed that the clone encoded a functional human GLP-2R protein, which led to cAMP production in COS cells in response to GLP-2 treatment (Figure 8).

EXAMPLE 8 Antibodies directed to the GLP-2 receptor

1. Antipeptide antibodies

Antipeptide antibodies were raised in rabbits against an N-terminal peptide (QTRENTTDIWQDESE), a C-terminal peptide (SEGDGSETLQKLR) and extracellular loop 1(SHNSUSKRPDDESG) of the rat GLP-2 receptor.

Immunocytochemical analysis of serum produced as above confirmed that serum contained antibodies directed to the GLP-2 receptor which do not cross react with the rat GLP-1 receptor.

2. Antibodies to a GLP-2 receptor raised against a fusion protein

Polynucleotide encoding the C-terminal region of rat GLP-2 receptor (amino acids 444-550) was spliced to the C-terminus of glutathione S-transferase (GST) in pGEX-2T and expressed in E.coli strain SURE1. Protein was purified using affinity chromatography using the above GLP-2 C-terminal fragment fused to the C-terminus of maltose binding protein. Protease degradation was minimized by using a cocktail of protease inhibitors (Boeringer Mannheim).

Antibodies were raised generally according to the method disclosed in Antibodies: A laboratory manual, Harlow and Lane, Cold Spring Harbor Laboratory, 1988. Briefly, the

5 GLP-2-GST fusion protein was used to raise antibodies in rabbits as follows. Initial injection was with 100 µg of fusion protein in complete Freund's adjuvant at multiple sites, intramuscularly and subcutaneously. Booster injections were made at multiple sites intramuscularly with 100 µg of fusion protein in incomplete Freund's adjuvant at days 14, 21, 42 and 56.

Antisera was affinity purified using a GLP-2-MBP fusion protein affinity column. Immunocytochemical analysis confirmed that these antibodies specifically recognize the GLP-2 receptor.

EQUIVALENTS

10 The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. Indeed, various modifications of the above-described makes for carrying out the invention which are obvious to those skilled in the field of biochemistry, molecular biology or related fields are intended to be within the scope of the following claims.

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We claim:

1. An isolated polynucleotide which codes for the human GLP-2 receptor having the amino acid sequence of amino acids 67-553 of SEQ ID NO: 12.

5 2. The isolated polynucleotide according to claim 1, comprising nucleotides 318-1780 of SEQ ID NO: 11.

10 3. An isolated polynucleotide which codes for the rat GLP-2 receptor having the amino acid sequence of amino acid 67-550 of SEQ ID NO:2.

15 4. The isolated polynucleotide according to claim 3, having nucleotides 335-1789 of SEQ ID NO: 1.

20 5. An isolated polynucleotide which hybridizes under conditions of high stringency with a polynucleotide defined in claim 2 or claim 4.

25 6. An oligonucleotide comprising at least 15 nucleotides and having a polynucleotide sequence that hybridizes under conditions of high stringency to a polynucleotide according to any of claims 1-5.

30 7. An oligonucleotide according to claim 6, wherein the nucleotide sequence of the oligonucleotide corresponds identically with a region of the polynucleotide defined in claim 2 or claim 4.

8. An oligonucleotide according to claim 6, wherein the nucleotide sequence of the oligonucleotide corresponds identically with a region common to the polynucleotides defined in claim 2 and claim 4.

9. In labelled form, a polynucleotide selected from a polynucleotide as defined in any of claims 1-5, and an oligonucleotide as defined in any of claims 6-8.

10. A recombinant polynucleotide comprising a GLP-2 receptor-encoding polynucleotide as defined in any of claims 1-5, and expression controlling elements linked operably therewith to drive expression thereof.

5 11. A cell that has been genetically engineered by the incorporation expressibly therein of a polynucleotide according to any of claims 1-5.

12. The cell according to claim 10, which is a mammalian cell.

10 13. An antibody which selectively binds to a GLP-2 receptor coded for by the polynucleotide according to any of claims 1-5.

14. A recombinant GLP-2 receptor comprising amino acids 67-553 of SEQ ID NO:12.

15 15. A recombinant GLP-2 receptor comprising amino acids 67-550 of SEQ ID NO:2.

20 16. A method for identifying GLP-2 receptor ligands, which comprises the steps of:

- (1) incubating a candidate ligand with a cell as defined in claim 11 or with a membrane preparation obtained therefrom; and then
- (2) determining whether binding between the GLP 2 receptor and the candidate ligand has occurred.

1 AAGCTTCGCG GCCTCTGCAG AKGACTTGTG CAAACACTTC CTCTCTGGAC
51 AAGGAGGAAT GCAGGAGGCC ACCGCCTGCA GTACATCTTG GAGTGTGGGA
101 GGGATGTGCC TGCACTTGTG AACGGGCGCC AGGAGAATGA GGCCCCAACC
151 AAGCCCCGCA GTGCCCAGTA GATGCAGAGA GGCACCCGTG CCCCAGTGTA
201 GGGCACAGCC AGTGGGCATC CCTGAGGCCC AGGGGCCCCGT TCCTCTCCAC
251 TCCCAACAGA TCGTCTGCT GTGGGGCCCT GGGAGGCCCT TCCTCGCCCT
301 GCTTCTGCTG GTTCCATCA AGCAAGTTAC AGGATCGCTC CTCAAGGAGA
351 CAACTCAGAA GTGGGCTAAT TATAAGGAGA AGTGTCTGGA AGACTTGCAC
401 AATAGACTTT CTGGCATATT TTGTAATGGG ACATTTGATC GGTATGTGTG
451 CTGGCCTCAT TCTTATCCTG GAAATGTCTC TGTTCCCTGT CCTTCATACT
501 TACCTTGGTG GAATGCAGAG AGCCCAGGAA GGGCCTACAG AACTGCTTG
551 GCTCAGGGGA CTTGSCAGAC GCGAGAGAAC ACCACAGATA TTTGGCAGGA
601 TGAATCAGAA TGCTCAGAGA ACCACAGCTT CAGACAAAAC GTGGATCACT
651 ACGCCTTGCT ATACACCTTG CAGCTGATGT AACTGTGGG CTACTCCGTG
701 TCTCTCATCT CCTCTCTCTT GGCTCTTACA CTCTCTTGT TCCTTCGAAA
751 ACTGCATTGC ACACGCAATT ACATCCACAT GAACCTGTTT GCTTCGTTCA
801 TCCTGAAAGT TCTGGCTGTC CTGGTGAAGG ACATGGTCTC CCACAACTCT
851 TACTCCAAGA GGCCCGATGA TGAGAGTGGA TGGATGTCAT ATCTGTCAGA
901 GACATCCGTC TCCTGTGCTC CCGTCCAGGT CCTCCTGCAC TACTTTGTGG
951 GCACCAATCA CTTGTGGCTG CTGGTTGAAG GACTTTACCT CCACACTCTG
1001 CTGGAGCCCA CAGTGTTTCC TGAAAGGCGG CTGTGGCCCA AGTACCTGGT
1051 GGTGGGTTGG GCCTTCCCCA TGCTGTTTGT TATTCCTGG GGTTTTGCCC
1101 GTGCACACCT GGAGAACACA CGGTGCTGGG CCACAAATGG GAACCTGAAA
1151 ATCTGGTGGA TCATCAGAGG ACCCATGCTG CTTTGTGTAA CAGTTAATTT
1201 CTTTCTCTTC CTCAAGATTC TCAAGCTTCT CATTTCTAAG CTCAAAGCTC
1251 ATCAGATGTG CTTCAGAGAC TACAAATACA GATTGGCGAA ATCAACGTTG
1301 CTCCTCATTC CTTTGTGGG GGTTCATGAG GTCCTCTTCA CTTTCTTCCC
1351 CGACGACCAA GTTCAAGGAT TTTCAAACG TATTCGACTC TTCATCCAGC
1401 TGACACTGAG CTCTGTCCAC GGATTCTGG TGGCCTTGCA GTATGGCTTT

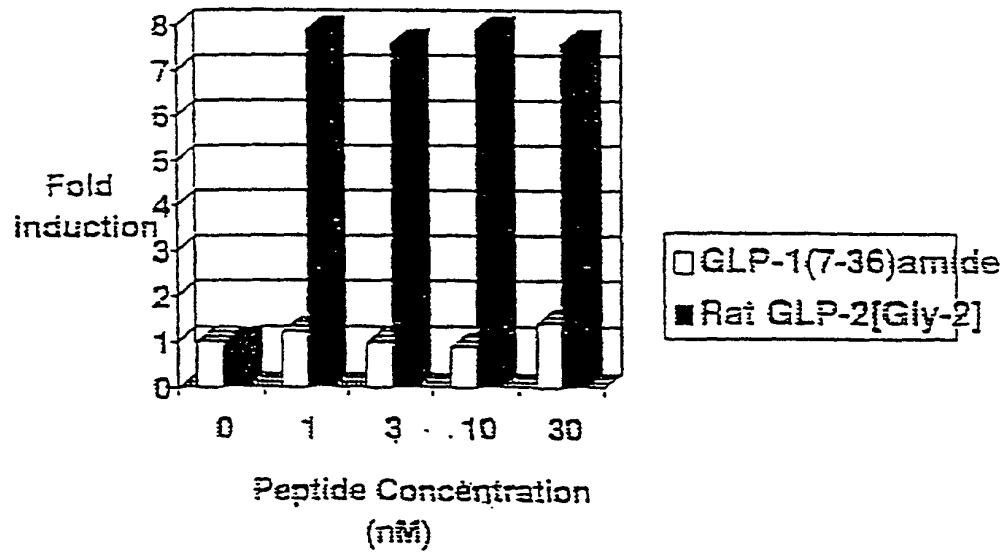
1451 GCCAATGGAG AGGTGAAGGC AGAGCTGCGA AAGTCATGGG GCCGCTTCTT
 1501 ATTAGCCCCG CACTGGGGCT GCAGAACCTG TGTCTGGGG AAGAATTTC
 1551 GGTTCCTGGG GAAGTGTTC AAGAAGCTGT CGGAGGGAGA TGGCTCTGAG
 1601 ACACTCCAGA AGCTGCGGTT CTCCACATGC AGCTCACACC TGGCCTCTGA
 1651 GACCCTGGGA GACGTGGGG TACAGCCTCA CAGGGGCCGT GGAGCTTGGC
 1701 CCCGGGGAAG CAGCCTGTCT GAGAGCAGTG AGGGAGACTT CACCCTGGCC
 1751 AATACGATGG AGGAGATTCT GGAAGAGAGT GAGATCTAAG GCAGGGTCCA
 1801 TCACCGCAGC TTGGCCACTG ARGAmCCAAC CcTARGAAGG ATKTTGCCGA
 1851 RCCCARGGTC CTCCTCTTCC TATGTWctAT MCCCATTTTG ATGTGAAGTC
 1901 TCTCCTGGGT GAMCAASCTC TGTACCAACS ARTCTCagTC CCTCTTGCCC
 1951 TTGTCAcCCT ACTACCCCTC CCCCATCaCa CATgTTTTCC AGAATKTCCG
 2001 TTGTTTTGGG GGGGGGGGTC TTGCCCTAAA TTCAAGTsGA GTGGARCCCA
 2051 CCATGAAGAA AARTCATTTA TTAAATAGAR TCCGGTTAGG ATCTCCTTCC
 2101 CGTTCATGGT GCATGGCCTC CTTCCAAGGG ATGGGAGTCG GSTGCACTGG
 2151 AACCCACAG GAAAcTTTGA AGTATCCAGT TCTAGGGAAT TATAGCCAAT
 2201 ATTCTGAGAG AGCAAGTCTG AGATGAGAKC CGAGAATWGC AAGTGTWGA
 2251 CAWGCATTCA AGGAAACTCC TCACCTTTgG GCGAAACCTA tGGCAGGATc
 2301 GGCAtGGAGC AGCTATMTG CAAYGGCCGC TCACCTGGGA CATACCACTC
 2351 TCCTTGGGCA GGATGTGACC CCATGTKGTC CCCAGACTC CTCTCCTCCT
 2401 TGCTTSTSTT CYTTTCCYGT CAAGTCTCAC CTCCCTTTCT ACATCTCAGT
 2451 TCWGTTTGGT GTYGACAGAA GYYTGAATGT CACAATACTG CATGTGTTAG
 2501 TTTCTGTCGT CATTGCTGTG TCCAAATACC TGACCAGGAC CAATTTAAGC
 2551 GAGGAACTGC TACATGGGCG GCCGC

FIGURE 1

1 MRPQPSPAVP SRCREAPVPR VRAQPVGIPE AQGPVPLHSQ QMRLLWGPGR
51 PFLALLLLVS IKQVTGSLK ETTQKWANYK EKCLEDLHNR LSGIFCNGTF
101 DRYVCWPHSY PGNVSVPCPS YLPWWNAESP GRAYRHCLAQ GTWQTRENTT
151 DIWQDESECS ENHSFRQNV D HYALLYTLQL MYTVGYSVSL ISLFLALTLF
201 LFLRLKLCR NYIHMNLFAS FILKVLAVLV KDMVSHNSYS KRPDDESGWM
251 SYLSETSVSC RSVQVLLHYF VGTNHLWLLV EGLYLHTLLE PTVFPERRLW
301 PKYLVVGWAF PMLFVIPWGF ARAHLENTRC WATNGNLKIW WIIRGPMLLC
351 VTVNFFIFLK ILKLLISKLK AHQMCFRDYK YRLAKSTLLL IPLLGVEVL
401 FTFFPDQVQ GFSKRIRLFI QLTLSVHGF LVALQYGFAN GEVKAELRKS
451 WGRFLLARHW GCRTCVLGKN FRFLGKCSKK LSEG DGSETL QKLRFTCSS
501 HLAETLGDV GVQPHRGRGA WPRGSSLSES SEGDFTLANT MEEILEEESI

FIGURE 2

Figure 3

cAMP Response of COS Transfected with Clone

[illegible]

S F S L I S L F L A L T L L L F L R K L
 1 TCGTTCTCTCTATCTCCCTCTTCTCTGGCTCTCAAGCTCTCTTGTCTTCTCGAAAACCTC 61
 H C T R N Y I H M N L F A S F I L R T L
 61 CACTGCACGGCGCACTACATCCACATGAACTTGTCTTCTCTTCACTCTGAGAACCTCTG 120
 A V L V K D V V F Y N S Y S K R P D N E
 121 GCTGTACTGGTGAAGGACGTCGTCTTCTACAACTCTTACTCCAAGAGGCTTGACAATGAG 180
 N G W M S Y L S E M S T S C R S V Q V L
 181 AATGGGTGGATGTCCTACCTGTCAAGAGATGTCCACCTCTGCGGCTCACTCCAGGTTCTC 240
 L H Y F V G A N Y L W L L V E G L Y L H
 241 TTGCATTACTTTGTGGGTGCCAATTACTTATGCGTCTGCTTGAAGGCTCTACCTCCAC 300
 T L L E P T V L P E R R L W P K Y L L L
 301 ACGCTGCTGGAGCCCACTGCTTCTCTGAGAGGCGGCTGTGCGCCCAATACCTGCTGTTG 360
 G W A F P V L F V V P W G F A R A H L E
 361 GGTGGGGCTTCCCTGTGCTATTGTGTACCCCTGGGCTTGGCCCTGCAACCTGGAR 420
 N T G C W T T N G N K K I W W I E R G P
 421 AACACAGGGTGTCTGGACAACTAATGGGAATAAGAAATCTGCTGGATCATCCGAGGACCC 480
 M H L C V T V N F F I F L H I L K L L I
 481 ATGATGCTCTGTGTACAGTCAATTTCTTCATCTTCTGAAAATTCTCAAGCTTCTCAT 540
 S K L K A H Q M C F R D Y K Y R L A K S
 541 TCTAAGCTCAAGCTCATCAATGTGCTTCAGAGATTATAAATACAGATTGGCAATCA 600
 T L V L I P L L G V H E I L F S F I T D
 601 ACACTGGTCCCTCATTCCTTTATTGGGCGTTCAAGATCCTCTCTCTTTTCATCACTGAT 660
 D Q
 661 GATCAG 667

Figure 5

(SEQ ID NO: 11)

1 TGGAGAGGATTGTGCAAAACATTTCTTCTGTGGACCAAGAGGAATGCAAGAGGAGGCTGC 50
 61 CTGCGGTGCATCTTGGACGGCTAGAGAGATGTACCCCTACTTGTGAAGGTGCACGAGGAA 120
 121 M K L G S S R A G P G R G S A G L L P G 130
 GATGAAGCTGGGATCGAGCAGGGCAGGGCCTGGGAGAGGAAGCGCGGGACTCCTGCCTGG
 181 V H E L P M G I P A P W G T S P L S F H 240
 CGTCCACGAGCTGCCCATGGGCATCCCTGCCCTTGGGGGACCAGTCTCTCTCTCTTCCA
 241 R K C S L W A P G R P F L T L V L L V S 300
 CAGGAAGTGCTCTCTCTGGGCCCCCTGGGAGGCCCTTCTCACTCTGGTCTGTGGTTTC
 301 I K Q V T G S L L E E T T R K W A Q Y K 350
 CATCAAGCAAGTTACAGGATCCCTCCTTGAGGAACGACTCGGAAGTGGGCTCAGTACAA
 361 Q A C L R D L L K E P S G I F C N G T F 420
 ACAGGCATGTCTGAGAGACTTACTCAAGGAACCTTCTGGCATATTTTGTAAACGGGACATT
 421 D Q Y V C W P H S S P G N V S V P C P S 480
 TGATCAGTACGTGTGTGGCCTCATCTTCTCTGGAAATGTCTCTGTACCCTGGCCTTC
 481 Y L P W W S E E S S G R A Y R H C L A Q 540
 ATACTTACCTTGGTGGAGTGAAGAGAGCTCAGGAAGGGCCTACAGACACTGCTTGGCTCA
 541 G T W Q T I E N A T D I W Q D D S E C S 600
 GGGGACTTGGCAGACGATAGAGAACGCCACGGATATTTGGCAGGATGACTCCGAATGCTC
 601 E N H S F K Q N V D R Y A L L S T L Q L 550
 CGAGAACCACAGCTTCAAGCAAAACGTGGACCGTTATGCCTTGCTGTCAACCTTGCAGCT
 661 M Y T V G Y S F S L I S L F L A L T L L 720
 GATGTACACCGTGGGATACTCCTTCTCTCTTATCTCCCTCTTCTGGCTCTCACCTCCT
 721 L F L R K L H C T R N Y I H M N L F A S 780
 CTTGTTTCTTCGAAAACCTCCACTGCACGCGCAACTACATCCACATGAACTTGTGTTCTC
 781 F I L R T L A V L V K D V V F Y N S Y S 840
 TTTCTCTGAGAACCCTGGCTGTACTGGTGAAGGACGTCGTCTTCTACAACCTCTTACTC
 841 K R P D N E N G W M S Y L S E M S T S C 900
 CAGAGGCCTGACAATGAGAATGGGTGGATGTCTTACCTGTGAGAGATGTCCACCTCCTG
 901 R S V Q V L L H Y V G A N Y L W L L V 950
 CCGCTCAGTCCAGGTTCTCTTGCACTACTTGGGGTGCCAATTACTTATGGCTGCTGGT
 961 E G L Y L H T L L E P T V L P E R R L W 1020
 TGAAGGCCTCTACCTCCACACGCTGCTGGAGCCCACTGCTTCTGAGAGGCGGCTGTG

FIGURE 6A

	P R Y L L L G W A F P V L F V V P W G F	
1021	GCCCAGATACCTGCTGTTGGGTTGGGCTTCCCTGTGCTATTGTGTACCTGGGGTTT -----+-----+-----+-----+-----+	1030
1081	A R A H L E N T G C W T T N G N K K I W CGCCCGTGACACCTGGAGAACACAGGGTGCTGGACAACAAATGGGAATAGAAAATCTG -----+-----+-----+-----+-----+	1140
1141	W I I R G P M M L C V T V N F F I F L K GTGGATCATCCGAGGACCCATGATGCTCTGTGTAAACAGTCAATTTCTTCATCTTCTGAA -----+-----+-----+-----+-----+	1200
1201	I L K L L I S K L K A H Q M C F R D Y K AATTCTCAAGCTTCTCATTCTAAGCTCAAAGCTCATCAAATGTGCTTCAGAGATTATAA -----+-----+-----+-----+-----+	1260
1261	Y R L A K S T L V L I P L L G V H E I L ATACAGATTGGCAAATCAACACTGGTCCTCATTCTTTATTGGGCGTTTCATGAGATCCT -----+-----+-----+-----+-----+	1320
1321	F S F I T D D Q V E G F A K L I R L F I CTTCTCTTTTCATCACTGATGATCAAGTTGAAGGATTGTGAAAACCTATACGACTTTTCAT -----+-----+-----+-----+-----+	1380
1381	Q L T L S S F H G F L V A L Q Y G F A N TCAGTTGACACTGAGCTCCTTTCATGGGTTCTCGGTGGCCTTGACAGTATGTTTTGCCAA -----+-----+-----+-----+-----+	1440
1441	G E V K A E L R K Y W V R F L L A R H S TGGAGAAGTGAAGGCTGAGCTGCGGAAATACTGGGTCCGCTTCTTGCTAGCCCGCCACTC -----+-----+-----+-----+-----+	1500
1501	G C R A C V L G K D F R F L G K C P K K AGGCTGCAGAGCCTGTGTCTCTGGGGAAGGACTTCCGGTTCCTAGGAAAATGTCCCAAGAA -----+-----+-----+-----+-----+	1560
1561	L S E G D G A E K L R K L Q P S L N S G GCTCTCGGAAGGAGATGGCGCTGAGAAGCTTCGGAAGCTGCAGCCCTCACTTAACAGTGG -----+-----+-----+-----+-----+	1620
1621	R L L H L A M R G L G E L G A Q P Q Q D GCGGCTCCTACATCTAGCCATGCGAGGTCTTGGGGAGCTGGGCGCCAGCCCCAACAGGA -----+-----+-----+-----+-----+	1680
1681	H A R W P R G S S L S E C S E G D V T M CCATGCACGCTGGCCCCGGGGCAGCAGCCTGTCCGAGTGCAGTGAGGGGATGTCAACCAT -----+-----+-----+-----+-----+	1740
1741	A N T M E E I L E E S E I * GGCCAAACACCATGGAGGAGATTCTGGAAGAGAGTGAGATCTAGGGTGGAGTTCACCACC -----+-----+-----+-----+-----+	1800
1801	CTGGCTCTGCTCCCAGGGACTCTTGAGGGGGCCAGGAAGAGGAAGCAAAGCAGGACACA -----+-----+-----+-----+-----+	1860
1861	CGTTGCTGGGCACGGAATCATTCTCGTTCCATTACCATGCCACTTTGATATGAAAGCTA -----+-----+-----+-----+-----+	1920
1921	TCACAAGGTTCTTCAAGCTCTGTATGAAAGAGGCTGTGTGTCATGCTCAGCCCTCTGCC -----+-----+-----+-----+-----+	1980
1981	TGCTCTTCTCATCCTAATAACCCCCACCAGTGTGTTTTCCACAATGCCACCAGACCCTA -----+-----+-----+-----+-----+	2040
2041	GGGCCTGGCTCTAAATTCAAGCCAATGAAGTCCCACCCGGAATCTTTTTGCTTTTTACCC -----+-----+-----+-----+-----+	2100
2101	CTGGAAGAAATA -----+-----+-----+-----+-----+	2112

FIGURE 6B

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Human GLP-2 Receptor Complete Open Reading Frame

Note: Translation may start with M-1 or M-25.

Length: 553 April 21, 1997 07:42 Type: P Check: 2776

1 MXLGSSRAGP GRGSAGLLPG VHELPMGIPA PWGTSPLSFH RKCSLWAPGR
51 PFLTLVLLVS IKQVTGSLLE ETTRKWAQYK QACLRDLLKE PSGIFCNGTF
101 DQYVCWPHSS PGNVSVPCPS YLPWWSEESS GRAYRHCLAQ GTWQTENAT
151 DIWQDDSECS ENHSFKQNV DRYALLSTLQL MYTVGYSTSL ISLFLALTLL
201 LFLRKLHCTR NYIHMNLFAS FILRTLAVLV KDVVFYNSYS KRPDNENGWM
251 SYLSEMSTSC RSVQVLLHYF VGANYLWLLV EGLYLHTLLE PTVLPERRLW
301 PRYLLLGWAF PVLFFVWPWF ARAHLENTGC WTTNGNKKIW WIIRGPMMLC
351 VIVNFFIFLK ILKLLISKLK AHQMCFRDYK YRLAKSTLVL IPLGVHEIL
401 FSPITDDQVE GFAKLIRLFI QLTLSSTHGF LVALQYGFAN GEVKAELRKY
451 WVRFLLARHS GCRACVLGXD FRFLGKCPKX LSEGDGAEXL RKLQPSLNSG
501 RLLHLAMRGL GELGAQPQOD HARWPRGSSL SECSEGDVTM ANTMEEILEE
551 SEI

FIGURE 7

cAMP Response to GLP-2

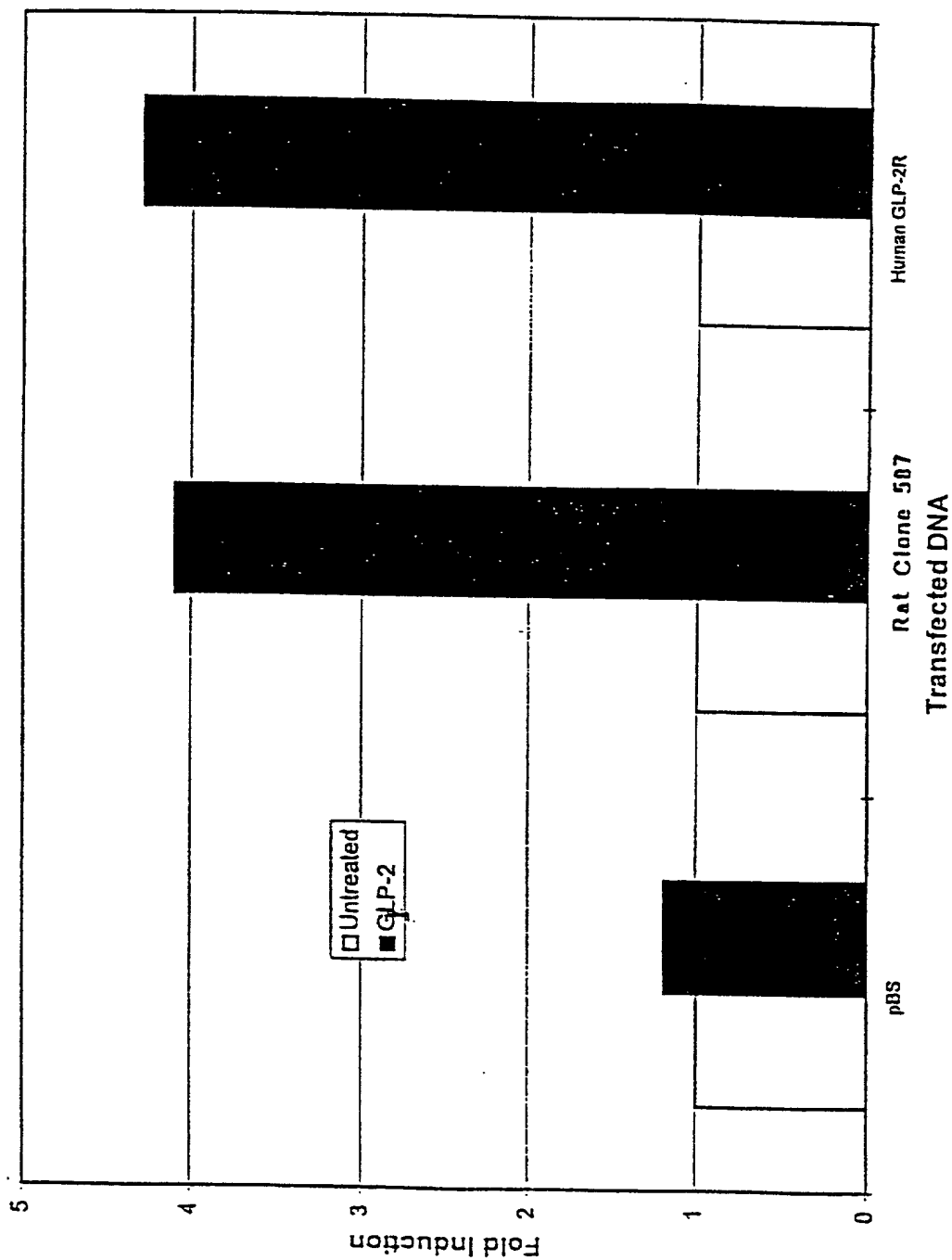


FIGURE 8

```

1 MRPQSPSPAVPSRCREAPVPRVRAQPVGIPAEAQGPVPLHSQQMRLWGPGR 50
|:| | | | | . | | | . | | | | | | | | | | |
1 MKLGSSRAGPGRGSAGLLPGVHELPMGIPAPWGTSPLSFHRKCSLWAPGR 50

51 PFLALLLLVSIKQVTGSLLKETTQKWANYKEKCLEDLHNRLSGIFCNGTF 100
| | | | . | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
51 PFLTLLVLLVSIKQVTGSLLLEETTRKWAQYKQACLRDLLKEPSGIFCNGTF 100

101 DRYVCWPHSYSPGNVSVPCPSYLPWWNAESPGRAYRHCLAQGTWQTRENTT 150
|. | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
101 DQYVCWPHSSPGNVSVPCPSYLPWWSEESSGRAYRHCLAQGTWQTIENAT 150

151 DIWQDESECSSENHSFRQNVDPHYALLYTLQLMYTVGYSVSLISLFLALTTF 200
| | | | : | | | | | | | | | | | | | | | | | | | | | | | | | | |
151 DIWQDDSECSSENHSFKQNVDRYALLSTLQLMYTVGYSFSLISLFLALTLL 200

201 LFLRKLHCTRNYIHMNLFASFILKVLAVLVKDMVSHNSYSKRPDDESGWM 250
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
201 LFLRKLHCTRNYIHMNLFASFILRTLAVLVKDVVFYNSYSKRPDNENGWM 250

251 SYLSETSVSCSRQVLLHYFVGTNHLWLLVEGLYLHTLLEPTVFPERRLW 300
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
251 SYLSEMSTSCRQVLLHYFVGANYLWLLVEGLYLHTLLEPTVLPERRLW 300

301 PKYLVVGWAFFPMLFVIPWGFARAHLENTRCWATNGNLKIWWIIRGPMMLC 350
| : | | . . | | | | . | | | : | | | | | | | | | | | | | | | | : | |
301 PRYLLLGWAFFPVLFVVPWGFARAHLENTGCWTTNGNKKIWWIIRGPMMLC 350

351 VTVNFFIFLKILKLLISKLKAHQMCFRDYKYRLAKSTLLLIPLLGVHEVL 400
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
351 VTVNFFIFLKILKLLISKLKAHQMCFRDYKYRLAKSTLVLIPLLGVHEIL 400

401 FTFFPDQVQGFESKRIRLFIQTLTSSVHGFLVALQYGFANGEVKAELRKS 450
|. | | | | : | | . | | | | | | | | | | | | | | | | | | | | | | |
401 FSFITDDQVEGEFAKLIRLFIQTLTSSFHGFLVALQYGFANGEVKAELRKY 450

451 WGRFLLARHWGCRTCVLGKNFRFLGKCSKLLSEGDGSETLQKLRFSTCSS 500
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
451 WVRFLLARHSGCRACVLGKDFRFLGKCPKKLSEGDGAEKLRKLQPSLNSG 500

501 ...HLASETLGDVGVPHRGRGAWPRGSSLSSESSEGDFTLANTMEEILEE 547
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
501 RLLHLAMRGLGELGAQPQODHARWPRGSSLSECSEGDVTMANTMEEILEE 550

548 SEI 550
| | |
551 SEI 553

```

FIGURE 9

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GL2R_RAT_R MRPQSPAVPSRCREAPVPRVRAQFVGIPFAQGFVPLHSQOMRLLWGPG-RPFLALLLLV
 HWBRPAT_TR MKLGSSRAGPGRGSAGLLPGVHELPMGIPAPWGTSPLSFHRKCSLWAPG-RPFLTLVLLV
 GLPR_HUMAN -----MAGAPGPLRLALLLLGMVGRAGPRP-----
 * * *

GL2R_RAT_R SIKQVTGSLLKETTKWANYKEKCLEDLHNRL---SGIFCNGTDFRYVCWPHSYPG-NVS
 HWBRPAT_TR SIKQVTGSLLLEETTRKWAQYKQACLRDLLEP---SGIFCNGTDFQYVCWPHSSPG-NVS
 GLPR_HUMAN ---QGATVSLWETVQKWREYRRCQQRSLTEDPPPATDLFCNRTFDEYACWPDGEPGSFVN
 * * * * *

GL2R_RAT_R VPCPSYLPWWNAESPGRAYRHCLAQGTWQTRENTTDIWQDESECSNHSFRQNVDRYALL
 HWBRPAT_TR VPCPSYLPWWSESSGRAYRHCLAQGTWQTIENATDIWQDDSECSNHSFKQNVDRYALL
 GLPR_HUMAN VSCPWYLPWASSVPQGHVYRFTAEGWLQKDNSSLPWRDLSECEESKRGERSSPEEQLL
 * * * * *

GL2R_RAT_R YTLQLMYTVGYSVSLISLFLALTFLFLRKLHCTRNYYHNMNLFASFILKVLAVLVKDMVS
 HWBRPAT_TR YTLQLMYTVGYSVSLISLFLALTFLFLRKLHCTRNYYHNMNLFASFILKVLAVLVKDMVS
 GLPR_HUMAN F-LYIIYTVGYALSFSALVIASAILLGFRLHCTRNYYHNMNLFASFILRALSVMFKDAAL
 * * * * *

GL2R_RAT_R HNSYSKRPDDESGWMSYLS-ETSVSCRSVQVLLHYFVGTNHLWLLVEGLYLHTLLEPTVF
 HWBRPAT_TR YNSYSKRPDNENGWMSYLS-EMSTSCRSVQVLLHYFVGANYLWLLVEGLYLHTLLEPTVL
 GLPR_HUMAN KWMYST-AAQOHQWDGLLSYQDLSLRLVFLMQYCVAAANYWLLVEGVLYTLTAFSVF
 * * * * *

GL2R_RAT_R PERRLWPKYLVVGWAFPMFLFVIPWGFARAHLENTRCWATNGNLKIWWIIRGPMMLCVTVN
 HWBRPAT_TR PERRLWPKYLVVGWAFPMFLFVIPWGFARAHLENTGCWTTNGNKKIWWIIRGPMMLCVTVN
 GLPR_HUMAN SEQWIFRLYVSIGWGVPLLFVVPWGVKLYLEDEGCWTRNSNMNYWLIIRLPILFAIGVN
 * * * * *

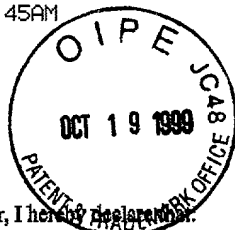
GL2R_RAT_R FFIFLKILKLLISKLAHQMCFRDYKYRLAKSTLLLIPLLGVEHLVFTFFPDQVQGFSG
 HWBRPAT_TR FFIFLKILKLLISKLAHQMCFRDYKYRLAKSTLVLIPLLGVEHLVFTFFPDQVQGFSG
 GLPR_HUMAN FLIFVRVICIVVSKLANLMCKTDIKCRLAKSTLTIPLLGTHEVIFAFVMDHARGTLR
 * * * * *

GL2R_RAT_R RIRLFIQTLTSSVHGFLVALQYGFANGEVKAELRKSWGRFLLARHWGCRTCVLGKGNFRFL
 HWBRPAT_TR LIRLFIQTLTSSFHGFLVALQYGFANGEVKAELRKYWVRFLARHSGCRACVLGKDFRFL
 GLPR_HUMAN FIKLFTLSFTSFQGLMVAILYCFVNNEVQLEFRKSWERWRLEHLHIQRDSSMKP-----
 * * * * *

GL2R_RAT_R GKCSKKLSEGDGSETLQKLRFTSCSS---HLASETLGDVGVPHRGRGAWPRGSSSLSESS
 HWBRPAT_TR GKCPKKLSEGDGAELKRLQPSINSGRLHLAMRGLGELGAQPPQDHARWPRGSSSLSECS
 GLPR_HUMAN LKCPSTSS-LSSGATAGSSMYTATCQASCS-----
 * * *

GL2R_RAT_R EGDFTLANTMEEIEESEI
 HWBRPAT_TR EGDVTMANTMEEIEESEI
 GLPR_HUMAN -----

FIGURE 10



3

PENNIE & EDMONDS DOCKET NO. 8607-023-999

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below at 201 et seq. underneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

CLONED GLUCAGON-LIKE PEPTIDE 2 RECEPTORS

and for which a patent application:

- ☐ is attached hereto and includes amendment(s) filed on _____ (if applicable)
- ☒ was filed in the United States on June 14, 1999 as Application Serial No. 09/331.127 (for declaration not accompanying application)
- with amendment(s) filed on _____ (if applicable)
- ☐ was filed as PCT international application Serial No. _____ on _____ and was amended under PCT Article 19 on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
			YES <input type="checkbox"/> NO <input type="checkbox"/>
			YES <input type="checkbox"/> NO <input type="checkbox"/>

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

APPLICATION NUMBER	FILING DATE

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED
08/845,546	April 14, 1997		X	
PCT/CA97/00969	December 15, 1997		X	

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	POST OFFICE ADDRESS	STREET <u>27 Wakefield Lane</u>	CITY <u>Waterdown</u>	STATE OR COUNTRY <u>Ontario, Canada</u>	ZIP CODE <u>L6R 2H3</u>
2-00 202	FULL NAME OF INVENTOR	LAST NAME <u>Gupta</u>	FIRST NAME <u>Ashwani</u>	MIDDLE NAME <u>K.</u>	
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	POST OFFICE ADDRESS	STREET <u>7031 Dunrobin Way</u>	CITY <u>Mississauga</u>	STATE OR COUNTRY <u>Ontario, Canada</u>	ZIP CODE <u>L5N 6Y4</u>
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4-00 204	FULL NAME OF INVENTOR	LAST NAME <u>McCallum</u>	FIRST NAME <u>Kirk</u>	MIDDLE NAME	
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5-00 205	FULL NAME OF INVENTOR	LAST NAME <u>Fan</u>	FIRST NAME <u>Ermei</u>	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY <u>Toronto</u>	STATE OR FOREIGN COUNTRY <u>Ontario, Canada</u> CAX	COUNTRY OF CITIZENSHIP <u>Canada</u>	
	POST OFFICE ADDRESS	STREET <u>20 Southport Street, Apt. #418</u>	CITY <u>Toronto</u>	STATE OR COUNTRY <u>Ontario, Canada</u>	ZIP CODE <u>M6S 4Y8</u>
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	POST OFFICE ADDRESS	STREET	CITY	STATE OR COUNTRY	ZIP CODE

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201 <u>Donald G. Munroe</u> DATE <u>99.8.17</u>	SIGNATURE OF INVENTOR 202 <u>Ashwani K. Gupta</u> DATE <u>Aug 17, 1999</u>	SIGNATURE OF INVENTOR 203 <u>T. Vyas</u> DATE <u>Aug 17/99</u>
SIGNATURE OF INVENTOR 204 <u>Kirk McCallum</u> DATE <u>99.8.30</u>	SIGNATURE OF INVENTOR 205 <u>Ermei Fan</u> DATE <u>Sept. 13, 1999</u>	SIGNATURE OF INVENTOR 206 <u>Ermei Fan</u> DATE